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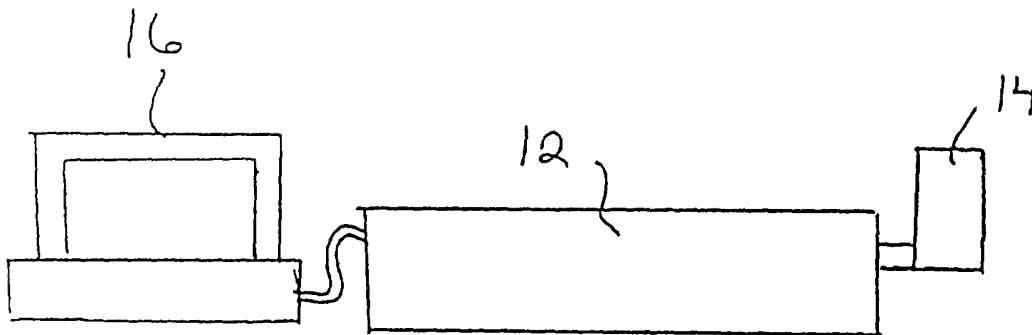
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(54) Title: METHOD OF NON-TARGETED COMPLEX SAMPLE ANALYSIS



(57) **Abstract:** A method for non-targeted complex sample analysis which involves the following steps. A first step involves providing a database (16) containing identifying data of known molecules. A second step involves introducing a complex sample containing multiple unidentified molecules into a Fourier Transform Ion Cyclotron Mass Spectrometer (12) to obtain data regarding the molecules in the complex sample. A third step involves comparing the collected data regarding the molecules in the complex sample with the identifying data of known molecules in order to arrive at an identification through comparison of the molecules in the sample.



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## METHOD OF NON-TARGETED COMPLEX SAMPLE ANALYSIS

## 5 FIELD OF THE INVENTION:

The present invention relates to a method of non-targeted complex sample analysis, with particular application to biology, and genomics in particular.

## BACKGROUND OF THE INVENTION

10 Functional genomics is an emerging field in biotechnology that focuses on the characterization of gene function. All organisms contain only one genotype. However, the expression of this genotype under varying developmental and environmental conditions results in an almost infinite number of possible phenotypes. It is the correlation of gene expression to phenotype that defines functional genomics. To properly study a gene we  
15 need to not only know its identity (i.e. sequence) but to be able to observe and characterize its expression patterns in response to developmental and environmental changes, in isolation as well as in relation to the other genes in the genome. To properly study the effects resulting from the expression of a gene we need to be able to characterize the phenotype resulting from this activity in an objective and quantifiable manner. This is  
20 what the non-targeted metabolic profiling technology invention described herein enables the functional genomics community to do.

The gene sequences of entire species are now known. Gene-chip technology has made it possible to monitor and quantify the changes in expression of each and every gene within the genome to developmental and environmental changes, simultaneously. Gene-  
25 chip technology is, in essence, non-targeted gene expression analysis even though it is, in actuality, a targeted analysis that just so happens to contain all of the possible targets. This is a powerful comprehensive capability, but it was made possible by the fact that the genome is a finite and unitary entity. The analogous phenotypic capability would be to have every metabolite and protein of an organism known and on a chip. This is not  
30 possible due to the fact that not only are there multiple phenotypes, but a virtually infinite number of metabolites and proteins are possible. To be complementary to the current state of genomic analysis, phenotypic analysis must be non-targeted in "actuality". The non-

targeted metabolic profiling technology described herein is the only platform that satisfies the requirements of non-targeted phenotypic analysis. Furthermore, this technology is not restricted to any one species, but is equally effective in all plant and animal species.

Deciphering the complex molecular makeup of an individual phenotype is a formidable task. To be able to accurately and reproducibly generate this phenotypic information in such a way that the virtually infinite number of possible phenotypes can be compared to one another and correlated to gene expression is the crux of the dilemma that faces functional genomics. On the molecular level, the phenotype of a given biological system can be divided into the proteome and the metabolome. Since gene expression results in protein synthesis, the proteome is the first and most direct link to gene expression. However, due to the complex interactions of metabolic pathways, it is difficult to predict the effects that changes in the expression levels of a given protein will have on the overall cellular processes that it may be involved in. The metabolome, on the other hand, is the summation of all metabolic (proteomic) activities occurring in an organism at any given point in time. The metabolome is therefore a direct measure of the overall or end effect of gene expression on the cellular processes of any given biological system at any given time. For this reason, the metabolome should prove to be the more powerful of the two phenotypes in actually understanding the effects of gene function and manipulation. The non-targeted metabolic profiling technology described herein is the only comprehensive metabolic profiling technology available.

Isolation, identification, and quantitation are the three fundamental requirements of all analytical methods. The primary challenge for a non-targeted metabolome analysis is to meet these requirements for all of the metabolites in the metabolome, simultaneously. The second and perhaps more difficult challenge is to be able to meet these requirements with sufficient throughput and long-term stability such that it can be used side by side with gene-chip technology. Such technology will drastically reduce the time that is required for the function of a particular gene to be elucidated. In addition, databases of such analyses enable very large numbers of phenotypes and genotypes to be objectively and quantitatively compared. There is no such product or technology available to functional genomics scientists at this time. The non-targeted metabolic profiling technology described herein has been extensively tested in multiple species. In all cases, the

technology has verified the metabolic variations known to exist between various genotypes and developmental stages of different species.

Key Technology Concept. The non-targeted metabolic profiling technology described herein can separate, quantify and identify all of the components in a complex biological sample quickly and simultaneously. This is achieved without any *a priori* selection of the metabolites of interest and is therefore unbiased. These data are exported to a database that allows the researcher to directly compare one sample to another (i.e. mutant vs. wild-type, flowering vs. stem elongation, drought stress vs. normal growing conditions, etc.) or to organize the entire database by metabolite concentration (i.e. which genotype has the greatest or least expression of a given metabolite). This technology is equally applicable to the study of human disease. To make use of this information, the researcher just types in the empirical formula(s) or the accurate mass(es) of the metabolite(s) he or she is interested in and the software will organize the data accordingly.

The ability to conduct an analysis of the composition of substances in biological samples is critical to many aspects of health care, environmental monitoring as well as the product development process. Typically the amount of a specific substance in a complex mixture is determined by various means. For example, in order to measure analytes in a complex mixture, the analyte(s) of interest must be separated from all of the other molecules in the mixture and then independently measured and identified.

In order to separate the analytes in a complex mixture from one another, unique chemical and/or physical characteristics of each analyte are used by the researcher to resolve the analytes from one another. These unique characteristics are also used to identify the analytes. In all previously published reports of complex mixture analysis, the methodologies require known analytical standards of each potential analyte before the presence and/or identity of a component in the unknown sample can be determined. The analytical standard(s) and the unknown sample(s) are processed in an identical manner through the method and the resulting characteristics of these standards recorded (for example: chromatographic retention time). Using this information, a sample containing unknown components can be analyzed and if a component in the unknown sample displays

the same characteristic as one of the known analytical standard (s), the component is postulated to be the same entity as the analytical standard. This is targeted analysis technology. Targeted analysis technology is one-way. The researcher can go from known standard to methodology characteristics but not from methodology characteristics to known standard. The researcher can only confirm or refute the presence and/or amount of one of the previously analyzed standards. The researcher cannot go from the method characteristics of an unknown analyte to its chemical identity. The major drawback of this type of analysis is that any molecule that was not identified prior to analysis is not measured. As a result, much potentially useful information is lost to the researcher. To be truly non-targeted, the method must allow the researcher to equally evaluate all of the components of the mixture, whether they are known or unknown. This is only possible if the defining physical and/or chemical characteristics of the analyte are not related to the method of analysis but are inherent in the composition of the analyte itself (i.e. its atomic composition and therefore its accurate mass).

#### Key benefits of non-targeted metabolic profiling technology

1. Multidisciplinary. Virtually only one set of analyses would need to be performed on a given sample and the data resulting from this analysis would be available to all scientists regardless of the area of research they are focusing on.
2. Comprehensive. The non-targeted approach assesses ALL metabolite changes and will thus lead to a faster and more accurate determination of gene function/disfunction.
3. Unknown Metabolite Discovery. The non-targeted approach has the potential of identifying key metabolic regulators that are currently unknown, and which would not be monitored in a targeted analysis scenario.
4. High Throughput. The system is can be fully automated and analysis time is short allowing 100's of samples to be analyzed per instrument per day.
5. Quantitative. The system is reproducible and has an effective dynamic range  $> 10^4$ . Relative changes in metabolite expression over entire populations can be studied.

Business Impact of Technology. The ability to generate searchable databases of the metabolic profiles of a given organism will represent a revolution in how the effects of genetic manipulation on a species can be studied. Currently our knowledge of the actual

genetic code is much greater than our knowledge of the functions of the genes making up this code. After the mapping of the genome, the next greatest challenge will be determining the function and purpose of these gene products and how manipulation of these genes and their expression can be achieved to serve any number of purposes. The time, energy, and cost of investigating the effects of genetic manipulation are great. A database that can be searched for multiple purposes and which contains direct measures of the metabolic profiles of specific genotypes has the potential to dramatically decrease the amount of time required to determine the function of particular gene products. Such a database will reduce the risk of investing a large amount of time and resources researching genes which may have effects on protein expression, but due to down-stream feedback mechanisms, no net effect on metabolism at the whole cell or organism level.

In an article published in CURRENT OPINION IN PLANT BIOLOGY in 1999 entitled "Metabolic Profiling: a Rosetta Stone for genomics?", Trethewey, Krotzky and Willmitzer indicated that exponential developments in computing have opened up the "possibility" of conducting non-targeted experimental science. While recognizing that it would not be possible to work with infinite degrees of freedom, the opinion was advanced that the power of post-experimental data processing would make possible this non-targeted approach. The non-targeted approach described in that article dealt only with the post acquisition analysis of metabolite data; not the non-targeted collection of metabolite data.

Thus the feasibility of non-targeted analysis of complex mixtures is neither obvious nor simple. The three major problems surrounding the non-targeted analysis of complex mixtures are: the ability to separate and identify all of the components in the mixture; the ability to organize the large amounts of data generated from the analysis into a format that can be used for research; and the ability to acquire this data in an automated fashion and in a reasonable amount of time.

## SUMMARY OF THE INVENTION

What is required is a method of non-targeted complex sample analysis.

5 According to the present invention there is provided a method for non-targeted complex sample analysis that involves the following steps. A first step involves providing a database containing identifying data of known molecules (this database contains the elemental compositions of all molecules previously identified in nature, organized by species, metabolic processes, subcellular location, etc.). A second step involves  
10 introducing a complex sample containing multiple unidentified molecules into a Fourier Transform Ion Cyclotron Mass Spectrometer to obtain data regarding the molecules in the complex sample. A third step involves comparing the collected data regarding the molecules in the complex sample with the identifying data of known molecules in order to arrive at an identification through comparison of the molecules in the sample. Molecules  
15 that are not represented in the database (i.e. unknowns) are automatically identified by determining their empirical formula. Thus, the method allows rapid identification of new molecules within the complex mixture related to specific molecules already identified, as well as identification of those molecules within the complex mixture that bear no relationship to those class or category of molecules already defined. As a result the  
20 analysis of complex mixtures is greatly simplified.

The invention, as described, uses the high resolving power of Fourier Transform Ion Cyclotron Mass Spectrometry (FTMS) to separate all of the components within the mixture that have different empirical formulas. This has been shown for petroleum  
25 distillates, but not for aqueous biological samples ionized in a "soft" ionization mode, where adduct ions can be problematic. The accurate mass capability of FTMS that enables the determination of empirical formula has been widely established. Furthermore FTMS is capable of performing high resolution/accurate mass 2D MS/MS which provides structural information that can be used to confirm the identities of components that have identical  
30 empirical formulas and allows the organization of metabolites based upon common structural components. This capability has been shown by isolated research groups but is not available on a commercial instrument. By integrating these capabilities with an

automated sample injection system and an automated data integration and database system, all of the components within a complex mixture can be analyzed rapidly and simultaneously. The data is then exported into a database that can be searched and organized by sample, or analyte. It is to be noted that unlike the approach advocated by Trethewey, Krotzky and Willmitzer, the present method is not dependant upon the advances in post experimental data processing. The non-targeted metabolic profiling technology described herein generates a dataset that is simple and compact. Computing technology capable of organizing and interpreting the described databases is readily available. No new advances are required. Furthermore, the technology does not have the finite limits inherent in the approach of Trethewey, Krotzky and Willmitzer.

#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings and figures, the drawings and figures are for the purpose of illustration only and are not intended to in any way limit the scope of the invention to the particular embodiment or embodiments shown, wherein:

FIGURE 1 is a side elevation view depicting non-targeted analysis of complex samples in accordance with the teachings of the present invention.

FIGURE 2 is an illustration of raw data (mass spectrum) collected from the FTMS showing how the metabolites in the complex mixture are separated from one another. Mass range displayed 100-350 amu.

FIGURE 3 is an illustration of raw data (mass spectrum) collected from the FTMS showing how the metabolites in the complex mixture are separated from one another. 10 amu mass range displayed.

FIGURE 4 is an illustration of raw data (mass spectrum) collected from the FTMS showing how the metabolites in the complex mixture are separated from one another. 1 amu mass range displayed.



FIGURE 5 is an illustration of raw data (mass spectrum) collected from the FTMS showing how the metabolites in the complex mixture are separated from one another. Mass range displayed 100-350 amu.0.1 amu window.

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FIGURE 6 is an illustration of strawberry pigment pathway (comparison of different developmental stages of an organism).

FIGURE 7 is an illustration of the extracted mass spectra of Phenylalanine from strawberry extracts from different developmental stages.

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FIGURE 8 is an illustration of the extracted mass spectra of Cinnamate from strawberry extracts from different developmental stages.

FIGURE 9 is an illustration of the extracted mass spectra of 4-Coumarate from strawberry extracts from different developmental stages.

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FIGURE 10 is an illustration of the extracted mass spectra of Naringenin from strawberry extracts from different developmental stages.

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FIGURE 11 is an illustration of the extracted mass spectra of Pelargonidin from strawberry extracts from different developmental stages.

FIGURE 12 is an illustration of the extracted mass spectra of Pelargonidin-3-glucoside from strawberry extracts from different developmental stages.

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FIGURE 13 is an illustration of glucosinolate mutants in *Arabidopsis thaliana* (comparison of genetic mutants to wild-type and identification of unknown metabolites). Relative changes in 3-Methylthiobutyl Glucosinolate illustrated.

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FIGURE 14 is an illustration of glucosinolate mutants in *Arabidopsis thaliana* (comparison of genetic mutants to wild-type and identification of unknown metabolites). Relative changes in 3-Methylsulphinylpropyl Glucosinolate illustrated.

- 5 FIGURE 15 is an illustration of glucosinolate mutants in *Arabidopsis thaliana* (comparison of genetic mutants to wild-type and identification of unknown metabolites). Relative changes in 3-Methylsulphinylheptyl Glucosinolate illustrated.

FIGURE 16 is an illustration of Tobacco Flower Analysis (Location of metabolite  
10 expected to be responsible for red color in tobacco).

FIGURE 17 is an illustration of Tobacco Flower Analysis (Location of unknown metabolite potentially involved in tobacco color).

- 15 FIGURE 18 is an illustration of Observed Metabolic Changes in Strawberry Development.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

- 20 The preferred method of non-targeted complex sample analysis embodiment will now be described with reference to FIGURE 1. The purpose of this invention is to provide a means of analyzing large numbers of complex samples, for example biological extracts, and be able to analyze the information in a non-targeted fashion after the analysis is complete to determine the differences between samples.

25

- In the invention complex samples are directly injected into the FTMS 12 though the use of an autosampler 14 with or without the additional use of a chromatographic column. The components of the mixture are ionized by one of many potential "soft" ionization sources (electrospray, APCI, FAB, SIMS, MALDI, etc.) and then transferred into the ion cyclotron  
30 resonance (ICR) cell with or without additional mass-selective pre-separation (quadrupole, hexapole, etc.). The ions are then separated and measured in the ICR cell with or without simultaneous MS/MS occurring. The data collected (mass spectrum) is integrated (the

mass, relative intensity, absolute intensity of each ion is determined) and processed, with or without calibration with known molecules of known concentrations. These data, with or without isotope elimination and empirical formula calculation, are then transferred to a database 16 that organizes and stores the data for future comparisons and functional  
5 analyses. Once stored in the database, individual samples can be compared with one another and those molecules that show different concentrations between the selected samples can be displayed. The entire database can be searched for specific molecules. The samples in the database can be listed from highest to lowest concentration or vice-versa. The molecules detected in the analysis can be compared with a database of known  
10 molecules and the molecules automatically identified. For molecules that do not match known molecules, the most likely empirical formulas can be displayed.

This approach provides numerous advantages to the researcher. There is a dramatic increase in the amount of information obtained from each sample (>10x compared to the  
15 most comprehensive targeted analysis procedure reported). Information is collected on both known and unknown components of a mixture. There is increased efficiency of data collection (data collection is approximately 10x faster than reported targeted analysis techniques). It provides a basis for unbiased comparison of unknown samples. Effects of gene modification on total cell metabolism can be determined instead of effects on only a  
20 small subset of metabolic processes (i.e. the relationship between different metabolic processes can be studied). By analyzing all metabolites the actual step within a metabolic process that is disrupted can be determined. Gene modifications that have an effect on protein expression but no net effect on cell metabolism can be identified. All of these analyses are completed simultaneously in one fast analysis, whereas multiple time-  
25 consuming analyses would have to be performed to get identical data at a tremendously higher cost.

Many examples exist for the use of FTMS for the analysis of complex mixtures, but none have introduced the concept of non-targeted analysis followed by database formation. The  
30 described method recognizes and utilizes some heretofore unused capabilities in FTMS. FTMS has the theoretical resolving power to separate all of the metabolites of different empirical formula in a complex biological sample. FTMS has the theoretical accurate

mass capabilities to assign empirical formulas to all of the metabolites in the complex biological sample. FTMS has the capability to perform 2 dimensional MS/MS on all of the metabolites in a complex biological sample. It is not necessary to know a priori what metabolites are present in a complex biological sample if the analytes could thus be separated and then be identified based upon their empirical formula and MS/MS fragment data and or by comparing them to a database of known analytes. Complex samples can be compared with one another to determine what analytes had different intensities between the samples. A database could be organized by analyte or by common MS/MS fragments. This approach significantly decreases the time and resources needed to elucidate gene function as a result of genetic manipulation, environmental changes, or developmental changes in an organism. One of the many applications of the described method invention include gene function determination in functional genomics research.

Numerous targeted LC-MS methods as well as other screening methods have been developed to analyze specific molecules or groups of molecules in complex samples. The major reason that this invention is novel and not obvious is because it employs a fundamentally different strategy for analytical analysis and is only possible with highly specialized instrumentation and methodology. Although the many independent theoretical research capabilities of FTMS have been known for at least 10 years, FTMS has only been used in a targeted way and for specialized research purposes. In the past 10 years no group has described the application of FTMS employed within the scope of the present invention. The present invention involves the combining of several theoretical FTMS capabilities into a comprehensive, non-targeted metabolic profiling procedure that has commercial utility in the analysis and interpretation of complex mixtures.

The method of the present invention comprises the following steps:

Generation of Known Metabolite Database. The identity (common name and empirical formula) and relevant biological information (species, metabolic processes involved in, cellular and subcellular location, etc) of all known biological metabolites are inputted into a commercial database program (i.e. Microsoft EXCEL, Table I.). The accurate

monoisotopic mass of these metabolites is automatically determined along with their  $[M+H]^+$  and  $[M-H]^-$  accurate mass ( $M+H$  and  $M-H$  refer to the mass of the metabolite when a proton ( $H^+$ ) is either added to the metabolite to create a positively charged ion or removed from the metabolite to create a negatively charged metabolite). The data  
5 collected from the FTMS analysis of the complex sample can then be compared to this database to immediately identify many of the components in the complex sample.

Preparation of samples for analysis. The metabolites are extracted from their biological source using any number of extraction/clean-up procedures that are typically used in  
10 quantitative analytical chemistry. Procedures are normally tailored to the source of the sample (i.e. leaf tissue, root tissue, blood, urine, brain, etc). For example, a 0.1g plant leaf sample may be extracted by placing it, 1.0 ml of 50/50 MeOH/0.1% formic acid, and 3 small glass beads in a test tube and then vortexing for one minute to homogenize the sample. The test tube is then centrifuged for 5 minutes. 100ul of the supernatant is then  
15 transferred from the test tube to a 96 well plate. The 96 well plate is placed upon the autosampler. 20ul of the supernatant is injected into the FTMS.

#### Typical operating conditions

Solvents. 50/50 MeOH/0.1% ammonium hydroxide as the mobile phase and for dilution  
20 for all negative ionization analyses and 50/50 MeOH/0.1% formic acid for all positive ion analyses.

Instrumentation. Bruker Daltonics APEX III Fourier Transform Mass Spectrometer (FTMS) equipped with a 7.0 Tesla actively shielded super conducting magnet with electrospray (ESI) and atmospheric chemical ionization (APCI) sources. ESI, APCI, and  
25 ion transfer conditions were optimized for sensitivity and resolution using a standard mix of serine, tetra-alanine, reserpine, HP Mix, and adrenocorticotrophic hormone fragment 4-10. Instrument conditions were optimized for ion intensity and broadband accumulation over the mass range of 100-1000 amu. One megaword data files were acquired and a sinm data transformation was performed prior to Fourier transform and magnitude calculations.

30 Calibration. All samples were internally calibrated for mass accuracy over the approximate mass range of 100-1000 amu using a mixture of the above-mentioned standards.

### Sample Analysis

Samples are introduced to the FTMS via an autosampler, or in some cases with a syringe pump. When the sample solution reaches the source of the FTMS (the source is where the  
5 FTMS ionizes the molecules in the sample solution), then molecules are ionized according to the principles of the particular ionization source used. The source can either be external to the mass analyzer or internal, depending on the type of ionization (for example in ESI and APCI ions are generated external to the mass analyzer and then transferred to the mass  
10 mass analyzer, whereas in electron impact ionization the molecules are ionized internal to the mass analyzer). The ions once generated and transferred (if necessary) to the mass analyzer are then separated and detected in the mass analyzer based upon their mass to charge ratio.

### Analyte Detection

All of the analytes within the complex mixture are analyzed simultaneously (see Figures 2-5). Structurally specific information (accurate mass with or without accurate MS/MS  
15 fragment masses) is obtained for all of the analytes without prior knowledge of the analyte's identity, and then this data is formatted in a way that is amicable to a  
20 comprehensive database.

### Complex Sample Database Formation

The typical process of database formation involves the following steps:

1. The output of the FTMS (calibrated mass spectrum) is filtered to remove all  
25  $^{13}\text{C}$  isotopes and peaks that have mass defects that do not correspond to singly charged biological metabolites;
2. Each of the peaks in this filtered peak list is then analyzed using the mass  
analysis program that is part of the instrument manufacturer's software package  
according to the elemental constraints provided by the researcher. This  
30 program returns all of the possible elemental compositions that are possible at a  
given mass within a certain selected error range.

3. Only the data (file name, sample ID, mass, relative intensity, absolute intensity, empirical formula(s)) from those peaks in the filtered peak list that satisfied the above constraints are exported to a final processed data file (Table II). Each sample analysis results in such a final processed data file.

4. Multiple databases can then be formed from the combining and comparing of the data files. Three such databases are:

a) Direct comparison of two samples to create a database of differences (Table VI);

b) Combination of multiple files to create a database capable of tracking changes through a series of samples (Table III);

c) Direct comparison of a whole series of samples to one control sample and then the combination of all the samples in the series into one database to allow comparisons within the series vs a common control (Figure 8).

The utility of the invention is illustrated in the following examples:

I. The ability to compare different developmental stages of an organism (Figures 6-12, Table IV).

In this example, we looked at the strawberry pigment pathway in strawberries. Figure 6 shows the full metabolic pathway. Figures 7-12 show the various metabolites in the pathway that we observed. It is to be noted that we were able to look at molecules of vastly different chemical compositions (amino acid, acid, flavenoid, glucoside). Here we were able to see the changes within a single genotype (red strawberry) as a function of developmental stage (green – white – turning – red) and compare it to a different genotype (white mutant). Only the non-targeted metabolic profiling technology described herein has this broad of a spectrum. Furthermore, as indicated in Table IV, these changes in the metabolome are directly correlated with changes in gene expression.

II. The ability to compare different genotypes (Figures 13-15, Table V).

In this example three different *Arabidopsis thaliana* mutants (TU1, TU3, TU5) that are known to have changes in the content and concentration of glucosinolates were compared

to a wild-type (WT). In this instance the non-targeted metabolic profiling technology described herein was able to confirm previous results as well as identify glucosinolate changes that had never before been observed.

5     III. The ability to detect and identify unknown metabolites involved in key pathways (Figures 16 and 17, Table IX).

In this example the flowers of a control (red) tobacco was compared to a white mutant. It was expected that the glucoside (Figure 16) was the metabolite responsible for color. However, when analyzed by the non-targeted metabolic profiling method, the expected  
10     metabolite was not observed. An unknown metabolite (Figure 17) was detected and identified (Table IX) to be the metabolite responsible for tobacco flower color.

IV. The ability to compare the effects of different environmental conditions on an organism (Table VI)

15     In this example the exuate from a carrot root grown under normal growing conditions (sufficient phosphate) was compared to the exuate from a carrot root grown under abnormal growing conditions (insufficient phosphate). Using non-targeted metabolic profiling we were able to identify key plant hormones that are excreted to promote symbiotic fungal growth under conditions of low phosphate.

20

V. The ability to group and classify metabolites based upon accurate MS/MS data (Table VII and Table VIII)

In this example accurate MS/MS fragmentation data was collected on the metabolites that were observed to be increased in the low phosphate conditions described above. Classes of  
25     molecules that have a similar substructure can be grouped together (in this case all metabolites with the C<sub>10</sub>H<sub>9</sub>N<sub>6</sub>O<sub>2</sub> fragment). This capability greatly enhances the ability to search and characterize different complex mixtures



VI. The ability to comprehensively monitor the metabolites of an organism (Table X, Figure 18)

In our study of the developmental stages of strawberry, we characterized the number of metabolites that we were observed as well as the number of metabolites that were observed to have changed in concentration between the different developmental stages. It is the comprehensive nature of this method that allows one to monitor and evaluate virtually all ongoing metabolic processes independently or in relation to one another. No other technology has this capability.

Table I Example of Known Metabolite Database

Common Name	Metabolic Process	Abbrev.	C	H	N	O	P	S	Monoisotopic Masses		
									M	M+H	M-H
glyoxylate			2	2		3			74.0004	75.0076	72.9932
Glycine		Gly,G	2	5	1	2			75.0320	76.0392	74.0248
pyruvic acid		PA	3	4		3			88.0160	89.0233	87.0088
L-Alanine		Ala,A	3	7	1	2			89.0477	90.0549	88.0404
Lactic Acid			3	6		3			90.0317	91.0389	89.0245
Cytosine			3	5	3	1			99.0432	100.0505	98.0360
Acetoacetic acid			4	6		3			102.0317	103.0389	101.0245
gamma aminobutyrate		GABA	4	9	1	2			103.0633	104.0705	102.0561
L-serine			3	7	1	3			105.0426	106.0498	104.0354
Histamine			5	9	3				111.0796	112.0869	110.0724
Uracil			4	4	2	2			112.0273	113.0345	111.0200
3-cyanoalanine			4	6	2	2			114.0429	115.0501	113.0357
L-Proline		Pro,P	5	9	1	2			115.0633	116.0705	114.0561
L-Valine		Val,V	5	11	1	2			117.0790	118.0862	116.0717
succinate			4	6		4			118.0266	119.0338	117.0194
L-Homoserine			4	9	1	3			119.0582	120.0655	118.0510
L-Threonine		Thr,T	4	9	1	3			119.0582	120.0655	118.0510
phosphoenolpyruvic acid		PEP	3	6		3	1		121.0054	122.0127	119.9982
L-cysteine		Cys, C	3	7	1	2		1	121.0197	122.0270	120.0125
Nicotinic Acid			6	5	1	2			123.0320	124.0392	122.0248
Thymine			5	6	2	2			126.0429	127.0501	125.0357
L-Isoleucine		Ile,I	6	13	1	2			131.0946	132.1018	130.0874
L-Leucine		Leu,L	6	13	1	2			131.0946	132.1018	130.0874
oxaloacetic acid		OAA	4	4		5			132.0059	133.0131	130.9986
L-asparagine		Asn,N	4	8	2	3			132.0535	133.0607	131.0462
L-Ornithine			5	12	2	2			132.0899	133.0971	131.0826
L-Aspartate		Asp,D	4	7	1	4			133.0375	134.0447	132.0303
Ureidoglycine			3	7	3	3			133.0487	134.0559	132.0415
L-malic acid			4	6		5			134.0215	135.0287	133.0143
Ureidoglycolate			3	6	2	4			134.0327	135.0400	133.0255
L-Homocysteine			4	9	1	2		1	135.0354	136.0426	134.0282
Adenine (Vitamin B4)			5	5	5				135.0545	136.0617	134.0473
Adenine			5	5	5				135.0545	136.0617	134.0473
3-Methyleneoxindole	Auxins		9	7	1	1			145.0528	146.0600	144.0455
Indolealdehyde	Auxins		9	7	1	1			145.0528	146.0600	144.0455
Indolenine epoxide	Auxins		9	7	1	1			145.0528	146.0600	144.0455
alpha-Ketoglutarate			5	6		5			146.0215	147.0287	145.0143
L-Glutamine		Gln,Q	5	10	2	3			146.0691	147.0763	145.0619
L-Lysine		Lys,L	6	14	2	2			146.1055	147.1127	145.0983
L-Glutamate		Glu,E	5	9	1	4			147.0531	148.0604	146.0459
L-Methionine		Met,M	5	11	1	2		1	149.0510	150.0583	148.0438
D-ribose			5	10		5			150.0528	151.0600	149.0456
Guanine			5	5	5	1			151.0494	152.0566	150.0422
Indole-3-acetotitrile	Auxins	IAN	10	7	2				155.0609	156.0681	154.0537

Comments: Any molecule of known chemical composition can be added to the database at any time. The database is comprised of accurate monoisotopic masses. All molecules that have a unique empirical formula will have a unique accurate mass. This mass is a constant and is independent of the methodologies discussed herein making it possible to analyze all  
5 of the components in a complex sample in a non-targeted fashion.

Figure 2 shows two raw mass spectrums. The top one is from the extract of a green stage strawberry and the lower one is from the extract of a red stage strawberry. Over 500  
10 unique chemical entities were observed over the mass range displayed above (100-350 amu; which is only a subset of the entire mass range analyzed (100-5000)). Figures 3, 4, and 5 show smaller and smaller mass ranges to illustrate the separation of the metabolites.

Figure 5 shows the resolution of the mass spectrum above 165,000. This extremely high  
15 resolution is necessary in order to separate all of the metabolites and thus be able to compare the two samples and determine the changes, if any.

Table II Illustration of processed data (file ID, mass, intensity, empirical formula, relative error)

FileID	Mass	Int	C	H	N	O	P	S	Err	C	H	N	O	P	S	Err
ESI_POS_pri_4_rs2_50_50	99.044061	2.05E+06	5	6	0	2	0	0	0.05							
ESI_POS_pri_3_ts_50_50	99.044082	1.33E+06	5	6	0	2	0	0	0.26							
ESI_POS_pri_3_ts_50_50	102.054929	2.56E+06	4	7	1	2	0	0	0.25							
ESI_POS_pri_1_gs_50_50	102.054956	3.08E+06	4	7	1	2	0	0	0.01							
ESI_POS_pri_2_vs_50_50	102.054962	1.36E+06	4	7	1	2	0	0	0.07							
ESI_POS_pri_4_rs2_50_50	104.070595	1.93E+06	4	9	1	2	0	0	0.10							
ESI_POS_pri_4_rs1_50_50	104.070624	1.75E+06	4	9	1	2	0	0	0.18							
ESI_POS_pri_5_gs_acn	104.106977	2.73E+06	5	13	1	1	0	0	0.13							
ESI_POS_pri_2_vs_50_50	104.106979	2.73E+06	5	13	1	1	0	0	0.11							
ESI_POS_pri_6_vs_acn	104.106981	1.84E+06	5	13	1	1	0	0	0.09							
ESI_POS_pri_1_gs_50_50	104.107	3.88E+06	5	13	1	1	0	0	0.09							
ESI_POS_pri_3_ts_50_50	106.049869	1.21E+08	3	7	1	3	0	0	0.01							
ESI_POS_pri_1_gs_50_50	106.04987	1.36E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_2_vs_50_50	106.04987	1.63E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_4_rs1_50_50	106.04987	1.08E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_4_rs2_50_50	106.04987	1.53E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_5_gs_acn	106.04987	2.59E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_6_vs_acn	106.04987	2.45E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_7_ts_acn	106.04987	2.62E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_8_rs1_acn	106.04987	2.48E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_8_rs2_acn	106.04987	2.33E+08	3	7	1	3	0	0	0.00							
ESI_POS_pri_6_vs_acn	107.070237	1.34E+06	4	10	0	3	0	0	0.31							
ESI_POS_pri_8_rs1_acn	107.070322	1.28E+06	4	10	0	3	0	0	0.48							
ESI_POS_pri_7_ts_acn	108.080743	2.79E+06	7	9	1	0	0	0	0.30							
ESI_POS_pri_4_rs2_50_50	109.028414	1.65E+06	6	4	0	2	0	0	0.07							
ESI_POS_pri_4_rs2_50_50	111.044016	1.41E+06	6	6	0	2	0	0	0.36							
ESI_POS_pri_8_rs2_acn	114.091316	2.74E+06	6	11	1	1	0	0	0.21							
ESI_POS_pri_1_gs_50_50	114.091319	3.02E+06	6	11	1	1	0	0	0.19							
ESI_POS_pri_4_rs1_50_50	114.091336	1.76E+06	6	11	1	1	0	0	0.04							
ESI_POS_pri_5_gs_acn	114.091337	3.87E+06	6	11	1	1	0	0	0.03							
ESI_POS_pri_2_vs_50_50	114.091342	2.70E+06	6	11	1	1	0	0	0.01							
ESI_POS_pri_7_ts_acn	114.091346	3.26E+06	6	11	1	1	0	0	0.05							
ESI_POS_pri_6_vs_acn	114.091358	3.18E+06	6	11	1	1	0	0	0.15							
ESI_POS_pri_8_rs1_acn	114.091375	2.74E+06	6	11	1	1	0	0	0.30							
ESI_POS_pri_4_rs2_50_50	114.091377	2.53E+06	6	11	1	1	0	0	0.32							
ESI_POS_pri_3_ts_50_50	114.091404	2.21E+06	6	11	1	1	0	0	0.56							
ESI_POS_pri_4_rs2_50_50	115.038958	3.43E+06	5	6	0	3	0	0	0.11							
ESI_POS_pri_5_gs_acn	115.038978	2.03E+06	5	6	0	3	0	0	0.07							
ESI_POS_pri_2_vs_50_50	115.038984	1.84E+06	5	6	0	3	0	0	0.12							
ESI_POS_pri_8_rs1_acn	115.038999	1.57E+06	5	6	0	3	0	0	0.25							
ESI_POS_pri_4_rs1_50_50	115.039032	1.86E+06	5	6	0	3	0	0	0.53							
ESI_POS_pri_3_ts_50_50	115.03905	1.67E+06	5	6	0	3	0	0	0.69							
ESI_POS_pri_2_vs_50_50	116.034226	1.76E+06	4	5	1	3	0	0	0.06							
ESI_POS_pri_1_gs_50_50	116.034233	2.43E+06	4	5	1	3	0	0	0.12							
ESI_POS_pri_3_ts_50_50	116.03425	2.07E+06	4	5	1	3	0	0	0.26							
ESI_POS_pri_1_gs_50_50	116.070538	2.60E+06	5	9	1	2	0	0	0.58							
ESI_POS_pri_3_ts_50_50	116.070601	1.46E+06	5	9	1	2	0	0	0.03							
ESI_POS_pri_2_vs_50_50	116.070643	1.46E+06	5	9	1	2	0	0	0.33							
ESI_POS_pri_4_rs1_50_50	118.086184	1.56E+06	5	11	1	2	0	0	0.60							
ESI_POS_pri_1_gs_50_50	118.086217	4.10E+06	5	11	1	2	0	0	0.32							
ESI_POS_pri_4_rs2_50_50	118.086231	1.52E+06	5	11	1	2	0	0	0.20							
ESI_POS_pri_2_vs_50_50	118.086234	1.23E+06	5	11	1	2	0	0	0.18							
ESI_POS_pri_3_ts_50_50	118.086246	2.74E+06	5	11	1	2	0	0	0.08							
ESI_POS_pri_5_gs_acn	118.086249	2.53E+06	5	11	1	2	0	0	0.05							

- 5 Comments: The mass spectrum is processed such that the  $^{13}\text{C}$  isotopes are first eliminated (this is only possible in FTMS analysis due to the high resolution and mass accuracy).

Then the remaining peaks are automatically analyzed using the mass analysis program that is included with the instrument using specific constraints chosen by the researcher (in the above example only those peaks that have the appropriate combination of carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulfur (S), or phosphorus (P) are returned). The final dataset now only contains monoisotopic, singly charged metabolites that have an accuracy of measurement of less than 1 ppm (err).

Table III Illustration of the database generated from the processed data;

Empirical Formula						Green Stage		White Stage		Turning Stage				Red Stage					
C	H	N	O	P	S	Mass	Int	Mass	Int	VS/GS	Mass	Int	TS/GS	TS/VS	Mass	Int	RS/GS	RS/VS	RS/TS
21	20	0	10	0	0	nf	1.30E+06	nf	1.30E+06	100	433.1130	1.68E+07	1292	1292	433.1128	2.98E+08	22923	22923	1774
25	34	6	19	0	0	nf	1.30E+06	723.1955	5.21E+07	4008	723.1952	1.12E+08	8615	215	723.1953	1.41E+08	10846	271	126
24	22	0	13	0	0	nf	1.30E+06	nf	1.30E+06	100	519.1132	3.16E+06	243	243	519.1133	1.21E+08	9308	9308	3829
22	32	6	1	0	0	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	397.2714	6.32E+07	4862	4862	4862
46	35	11	1	0	1	nf	1.30E+06	790.2821	2.62E+07	2015	790.2819	5.71E+07	4392	218	790.2822	4.54E+07	3492	173	80
19	17	11	3	0	0	nf	1.30E+06	448.1592	3.53E+07	2715	448.1591	4.88E+07	3754	138	448.1592	4.02E+07	3092	114	82
11	16	4	9	0	1	nf	1.30E+06	381.0710	1.68E+07	1292	381.0710	2.19E+07	1685	130	381.0709	2.75E+07	2115	164	126
9	18	8	5	0	3	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	415.0638	2.69E+07	2069	2069	2069
30	67	19	4	0	0	nf	1.30E+06	nf	1.30E+06	100	758.5697	3.27E+07	2515	2515	758.5698	2.44E+07	1877	1877	75
47	71	7	3	0	0	nf	1.30E+06	782.5697	3.67E+07	2823	782.5694	3.19E+07	2454	87	782.5697	2.12E+07	1631	58	66
22	40	14	5	0	2	nf	1.30E+06	645.2825	2.27E+07	1746	645.2823	2.71E+07	2085	119	645.2825	2.12E+07	1631	93	78
23	24	8	5	0	1	nf	1.30E+06	525.1667	4.15E+06	319	525.1663	1.54E+07	1185	371	525.1664	1.52E+07	1169	366	99
9	16	8	1	0	3	nf	1.30E+06	nf	1.30E+06	100	349.0683	1.42E+06	109	109	349.0685	1.50E+07	1154	1154	1056
20	28	4	11	0	1	nf	1.30E+06	533.1550	5.75E+06	442	533.1551	1.54E+07	1185	268	533.1550	1.38E+07	1062	240	90
22	29	3	1	0	3	nf	1.30E+06	448.1546	1.34E+07	1031	448.1545	1.73E+07	1331	129	448.1546	1.32E+07	1015	99	76
33	54	6	9	0	0	nf	1.30E+06	679.4031	1.52E+07	1169	679.4025	1.58E+07	1215	104	679.4028	1.31E+07	1008	86	83
14	29	3	13	0	0	nf	1.30E+06	448.1774	1.17E+07	900	448.1774	1.53E+07	1177	131	448.1774	1.28E+07	985	109	84
15	20	0	11	0	0	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	377.1078	1.24E+07	954	954	954
21	12	0	2	0	1	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	329.0634	1.17E+07	900	900	900
40	34	8	0	0	3	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	723.2143	1.13E+07	869	869	869
27	50	2	5	0	2	nf	1.30E+06	547.3240	1.21E+07	931	547.3239	1.22E+07	938	101	547.3240	1.06E+07	815	88	87
21	44	2	21	0	2	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	725.1951	1.05E+07	808	808	808
30	42	0	17	0	1	707.222203	5.04E+06	707.2220	1.94E+07	385	707.2216	5.34E+07	1060	275	707.2218	3.99E+07	792	206	75
12	24	4	11	0	1	nf	1.30E+06	nf	1.30E+06	100	nf	1.30E+06	100	100	433.1235	9.92E+06	763	763	763

Comments: In Table III, the data was sorted according to the relative expression of metabolites in the red stage vs the green stage of strawberry. The data can be organized by any field. What is observed is that the metabolite C<sub>10</sub>H<sub>20</sub>O<sub>10</sub> has a concentration that is at least 22923% of that observed in the green stage (this metabolite is not observed in the green stage so the value is a % of the background noise). This metabolite can be identified by its empirical formula as pelargonidin-3-glucoside, the primary pigment observed in strawberries that give them their red color. This process is automated.

Table IV Comparison of Metabolite and Gene Expression Data in Strawberry Color Formation (Red Stage vs. Green Stage)

Metabolic Pathway	Relative Metabolite Expression	Relative Gene Expression
4-Coumarate-COA to Naringenin Chalcone	4.3	3.3
Naringenin Chalcone to Naringenin	4.3	4.3
Leucopelargonidin to Pelargonidin	20*	6.7
Pelargonidin to Pelargonidin-3-Glucoside	42*	8.3

\*Reflects greater dynamic range of metabolic expression analysis

5

Comments: Figures 7 through 12 and Table IV show the power of non-targeted metabolic profiling in studying changes that occur during development. Non-Targeted metabolic profiling allows the researcher to monitor entire metabolic pathways simultaneously. There is no other methodology that allows for the simultaneous analysis of such a diverse range of analytes. All of the analytes illustrated above were extracted from the non-targeted data collected using the methodology and concepts presented in this application.

10

and identification of unknown metabolites). Relative changes in 3-Methylsulphinylheptyl Glucosinolate illustrated.

Table V Comparison of Glucosinolates in different *Arabidopsis thaliana* mutants

5

### *Arabidopsis Glucosinolate Mutants*

R=	Glucosinolates				
	WT	TU1	TU3	TU5	TU7
3-Methylthiobutyl	1.00	<0.06(nf)	2.69	0.14	0.36
3-Methylthiopentyl	1.00	<0.56(nf)	2.12	<0.56(nf)	0.71
3-Methylthioheptyl	1.00	1.00	<0.21(nf)	0.32	<0.21(nf)
3-Methylthiooctyl	1.00	2.93	<0.09(nf)	0.92	0.15
3-Methylsulphinylpropyl	1.00	27.62	1.37	21.56	0.37
3-Methylsulphinylbutyl	1.00	0.10	2.50	0.63	0.53
3-Methylsulphinylpentyl	1.00	1.56	3.11	0.79	1.11
3-Methylsulphinylheptyl	1.00	1.38	<0.37(nf)	0.64	<0.37(nf)
3-Methylsulphinylloctyl	1.00	6.16	<0.11(nf)	4.25	0.37
3-Indolylmethyl	1.00	4.44	0.90	1.85	0.71
Methoxy-3-indolylmethyl	1.00	1.41	0.67	0.59	0.46
C3H7OS	1.00 (nf)	>6.88	nf	nf	nf
C5H11O8S	1.00	2.68	0.73	0.85	0.60
C7H10O5S3	1.00 (nf)	>5.73	nf	>3.01	nf
C8H12O5S3	1.00	<0.37(nf)	1.95	<0.37(nf)	0.45
C13H26NO3S	1.00	2.55	1.05	1.18	0.44
C21H23O3	1.00	2.74	1.21	0.47	0.52

### 19 Glucosinolate Molecules Observed (17 reported)

Comments: In Table V, the applicability of the technology for comparing genetic mutants to their wild-type counterparts is illustrated. The non-targeted metabolic profiles of four mutants (TU1, TU3, TU5, and TU7) were compared to their wild-type counterpart. Here we show that not only can we identify and monitor the glucosinolates that had been previously analyzed using targeted analysis, but were able to identify previously unidentified glucosinolates. As is the case in all of our analyses, all of the other metabolites are also available for evaluation.

10

Table VI Illustration of database generated by directly comparing two samples (carrot root exuate in the presence and absence of phosphate) Summary of Metabolites that were Observed to be Increased in the -P Fraction

-P/+P Ratio (Corr.)	Mode	Minus P			Plus P		Proposed Empirical Formula										Observed As	Theoretical Mass	Error (ppm)
		Mass	Abs Int.	Corr. Int.	Mass	Abs Int.	C	H	N	O	P	S	Cl	Na	K	e'			
1172.550	ESI+	245.0783	2.35E+09	1.17E+09		1.00E+06	10	9	6	2						-1	+H	245.07815	0.73
1053.350	ESI+	467.1672	2.11E+09	1.05E+09		1.00E+06	22	23	6	6						-1	+H	467.1673589	-0.45
981.550	ESI+	177.0546	1.96E+09	9.82E+08		1.00E+06	10	9	3							-1	+H	177.0546206	-0.17
658.650	ESI+	223.0965	1.32E+09	6.59E+08		1.00E+06	12	15	4							-1	+H	223.0964854	-0.16
186.090	ESI+	261.0524	3.72E+08	1.86E+08		1.00E+06	12	14	4						1	-1	+K	261.0523672	0.05
73.375	ESI+	651.2412	1.47E+08	7.34E+07		1.00E+06	31	35	6	10						-1	+H	651.2409178	0.48
52.845	ESI+	328.1390	1.06E+08	5.28E+07		1.00E+06	15	22	1	7						-1	+H	328.1390785	-0.24
47.308	ESI+	619.2509	9.46E+07	4.73E+07		1.00E+06	31	35	6	8						-1	+H	619.2510885	-0.39
35.421	ESI+	559.3239	7.08E+07	3.54E+07		1.00E+06	28	43	6	6						-1	+H	559.3238596	0.13
34.279	ESI+	539.2613	6.86E+07	3.43E+07		1.00E+06	27	35	6	6						-1	+H	539.2612593	0.00
31.780	ESI+	307.0489	6.36E+07	3.18E+07		1.00E+06	12	19	3	3						-1	+H	307.049083	-0.60
28.136	ESI+	523.2299	5.63E+07	2.81E+07		1.00E+06	26	31	6	6						-1	+H	523.2299592	-0.09
25.510	ESI+	569.1988	5.10E+07	2.55E+07		1.00E+06	26	29	6	9						-1	+H	569.199053	-0.44
24.248	ESI-	279.1236	2.42E+07	2.42E+07		1.00E+06	15	19	5							1	-H	279.1237973	-0.60
22.393	ESI+	635.3554	4.48E+07	2.24E+07		1.00E+06	34	47	6	6						-1	+H	635.3551597	0.38
21.312	ESI+	543.3288	4.26E+07	2.13E+07		1.00E+06	28	43	6	5						-1	+H	543.3289449	-0.21
20.003	APCI+	377.1594	2.00E+07	2.00E+07		1.00E+06	20	25	7							-1	+H	377.1594796	-0.18
19.937	ESI+	291.0714	3.99E+07	1.99E+07		1.00E+06	11	15	9							-1	+H	291.0710585	1.04
15.314	APCI-	279.1239	1.53E+07	1.53E+07		1.00E+06	15	19	5							1	-H	279.1237973	0.26
13.322	ESI+	487.2663	2.86E+07	1.33E+07		1.00E+06	24	35	6	5						-1	+H	487.2663447	-0.07
13.273	ESI-	335.2227	6.63E+07	6.63E+07	335.2227	5.00E+06	20	31	4							1	-H	335.2227831	-0.40
13.091	APCI-	335.2230	1.60E+08	1.60E+08	335.2231	1.22E+07	20	31	4							1	-H	335.2227831	0.66
12.968	ESI+	242.0700	2.59E+07	1.30E+07		1.00E+06	15	20	10	9						-2	+2H	242.0701876	-0.86
11.693	ESI+	473.2507	2.34E+07	1.17E+07		1.00E+06	23	33	6	5						-1	+H	473.2506946	0.10
11.236	ESI-	167.6111	1.12E+07	1.12E+07		1.00E+06	18	29	3	3						2	-2H	167.6109945	0.33
9.001	ESI+	149.0233	4.81E+08	2.40E+08	149.0233	2.67E+07	8	5	3							-1	+H	149.0233204	0.00
8.226	ESI+	459.2352	1.65E+07	8.23E+06		1.00E+06	22	31	6	5						-1	+H	459.2350446	0.36
8.011	APCI+	319.2267	3.59E+07	3.59E+07	319.2267	4.48E+06	20	31	3							-1	+H	319.2267713	-0.22
7.742	ESI-	249.1494	2.14E+07	2.14E+07	249.1494	2.77E+06	15	21	3							1	-H	249.1496181	-0.71
7.279	ESI-	333.2071	1.43E+07	1.43E+07	333.2071	1.96E+06	20	29	4							-1	-H	333.207133	-0.13
7.163	ESI+	483.1415	1.43E+07	7.16E+06		1.00E+06	24	28	8						1	-1	+K	483.1415762	-0.12
6.902	ESI-	347.1864	1.15E+07	1.15E+07	347.1864	1.65E+06	20	27	5							1	-H	347.1863976	-0.11
6.655	APCI-	263.1290	6.66E+06	6.66E+06		1.00E+06	15	19	4							1	-H	263.1288827	0.26
6.270	APCI-	347.1867	1.87E+07	1.87E+07	347.1867	2.98E+06	20	27	5							1	-H	347.1863976	0.63
6.019	ESI+	345.1258	1.20E+07	6.02E+06		1.00E+06	14	22	6		1				1	-1	+K	345.1258237	-0.01
5.306	ESI-	263.1287	5.31E+06	5.31E+06		1.00E+06	15	19	4							1	-H	263.1288827	-0.69
5.300	ESI+	229.1047	1.06E+07	5.30E+06		1.00E+06	15	17			1					-1	+H	229.1045477	0.75
4.971	ESI-	191.1076	4.97E+06	4.97E+06		1.00E+06	12	15	2							1	-H	191.1077533	-0.80
4.603	ESI-	213.1494	2.32E+07	2.32E+07	213.1494	5.03E+06	12	21	3							1	-H	213.1496181	-1.02
4.600	ESI-	277.1443	4.60E+06	4.60E+06		1.00E+06	16	21	4							1	-H	277.1445327	-0.84
4.524	APCI-	333.2074	2.20E+07	2.20E+07	333.2075	4.87E+06	20	29	4							1	-H	333.207133	0.97
4.163	ESI-	199.1341	1.18E+07	1.18E+07	199.1341	2.83E+06	11	19	3							1	-H	199.1339681	0.61
3.392	ESI-	227.1650	3.17E+07	3.17E+07	227.1650	9.33E+06	13	23	3							1	-H	227.1652662	-1.05
3.131	ESI+	312.1441	6.26E+06	3.13E+06		1.00E+06	15	22	1	6						-1	+H	312.1441639	-0.08
3.111	APCI-	249.1497	1.54E+07	1.54E+07	249.1497	4.95E+06	15	21	3							1	-H	249.1496181	0.19
2.566	APCI-	329.2336	2.29E+07	2.29E+07	329.2335	8.92E+06	18	33	5							1	-H	329.2333477	0.58
2.438	ESI-	415.1794	2.44E+06	2.44E+06		1.00E+06	20	31	7		1					1	-H	415.1795976	-0.60
2.017	ESI+	285.0951	4.03E+06	2.02E+06		1.00E+06	10	17	6		2					-1	+H	285.0950624	-0.01

5

Comments: Table VI illustrates how our technology can be used to compare the metabolic profile of an organism under different environmental conditions. Here we were able to detect and identify key molecules involved in controlling the plant's response to phosphate conditions. This capability allows researchers to determine what effects changes in environmental conditions will have on the biological functions of an organism.

10

Table VII MS/MS Data for Selected Metabolites Observed to be Increased in the -P Fraction

Parent	Fragment	Loss Of:
C <sub>31</sub> H <sub>35</sub> N <sub>6</sub> O <sub>10</sub> [H <sup>+</sup> ] 651 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>10</sub> H <sub>9</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>9</sub> H <sub>7</sub> [H <sup>+</sup> ]	C <sub>12</sub> H <sub>12</sub> O <sub>5</sub> C <sub>12</sub> H <sub>14</sub> O <sub>6</sub> C <sub>21</sub> H <sub>24</sub> O <sub>8</sub>
C <sub>31</sub> H <sub>35</sub> N <sub>6</sub> O <sub>8</sub> [H <sup>+</sup> ] 619 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>10</sub> H <sub>9</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>9</sub> H <sub>7</sub> [H <sup>+</sup> ]	C <sub>12</sub> H <sub>12</sub> O <sub>3</sub> C <sub>12</sub> H <sub>14</sub> O <sub>4</sub> C <sub>21</sub> H <sub>24</sub> O <sub>6</sub>
C <sub>26</sub> H <sub>29</sub> N <sub>6</sub> O <sub>9</sub> [H <sup>+</sup> ] 569 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>10</sub> H <sub>9</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>9</sub> H <sub>7</sub> [H <sup>+</sup> ]	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub> C <sub>7</sub> H <sub>8</sub> O <sub>5</sub> C <sub>16</sub> H <sub>20</sub> O <sub>7</sub>
C <sub>28</sub> H <sub>43</sub> N <sub>6</sub> O <sub>6</sub> [H <sup>+</sup> ] 559 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>10</sub> H <sub>9</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>9</sub> H <sub>7</sub> [H <sup>+</sup> ]	C <sub>9</sub> H <sub>20</sub> O C <sub>9</sub> H <sub>22</sub> O <sub>2</sub> C <sub>18</sub> H <sub>20</sub> O <sub>4</sub>
C <sub>28</sub> H <sub>43</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] 543 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>10</sub> H <sub>9</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>9</sub> H <sub>7</sub> [H <sup>+</sup> ]	C <sub>9</sub> H <sub>20</sub> C <sub>9</sub> H <sub>22</sub> O C <sub>18</sub> H <sub>20</sub> O <sub>3</sub>
C <sub>27</sub> H <sub>35</sub> N <sub>6</sub> O <sub>6</sub> [H <sup>+</sup> ] 539 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>15</sub> H <sub>21</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>10</sub> H <sub>9</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ] C <sub>9</sub> H <sub>7</sub> [H <sup>+</sup> ]	C <sub>8</sub> H <sub>12</sub> O C <sub>8</sub> H <sub>14</sub> O <sub>2</sub> *C <sub>12</sub> H <sub>14</sub> O <sub>4</sub> C <sub>17</sub> H <sub>26</sub> O <sub>4</sub>
C <sub>26</sub> H <sub>31</sub> N <sub>6</sub> O <sub>6</sub> [H <sup>+</sup> ] 523 +ESI	C <sub>19</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub> [H <sup>+</sup> ] C <sub>19</sub> H <sub>21</sub> N <sub>6</sub> O <sub>4</sub> [H <sup>+</sup> ] *C <sub>14</sub> H <sub>17</sub> N <sub>6</sub> O <sub>2</sub> [H <sup>+</sup> ]	C <sub>7</sub> H <sub>9</sub> O C <sub>7</sub> H <sub>10</sub> O <sub>2</sub> *C <sub>12</sub> H <sub>14</sub> O <sub>4</sub>



	$C_{10}H_9N_6O_2[H^+]$ $C_9H_7[H^+]$	$C_{16}H_{22}O_4$
$C_{22}H_{23}N_6O_6[H^+]$ 467 +ESI	$*C_{10}H_9N_6O_2[H^+]$	$*C_{12}H_{14}O_4$
$*C_{12}H_{15}O_4[H^+]$ 223 +ESI	$*C_{10}H_9O_3[H^+]$ $C_9H_7O_3[H^+]$ $C_8H_5O_3[H^+]$ $C_6H_5O[H^+]$	$C_2H_6O$ $C_3H_8O$ $C_4H_{10}O$ $C_6H_{10}O_3$
$*C_{10}H_9O_3[H^+]$ 177 +ESI	$*C_8H_5O_3[H^+]$ $C_6H_5O[H^+]$	$C_2H_4$ $C_4H_4O_2$
$*C_8H_5O_3[H^+]$ 149 +ESI	$C_7H_5O_2[H^+]$ $C_6H_5O[H^+]$	$CO$ $C_2O_2$

Table VIII Determination of Metabolite Relations using MS/MS data

R1	R3	R2
$C_{10}H_8N_6O_2$	None	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_4H_8$	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_5H_{12}$	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_6H_6$	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_4H_6O_3$	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_9H_{10}O_2$	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_9H_{10}O_4$	$C_{12}H_{14}O_4$
$C_{10}H_8N_6O_2$	$C_6H_6$	$C_{12}H_{14}O_3$

Table IX. Mass Analysis of unknown peak observed in Tobacco Flower Analysis

*Mass Analysis of Unknown Peak*

Calibration Constants:  
 ML1: 108299134.679450  
 ML2: -16.576817  
 ML3: -2029.796744

Calibration Results:		
Ref. Masses	Exp. Masses	Diff (ppm)
124.039300	124.039298	0.0187
161.092070	161.092079	0.0542
303.166300	303.166272	0.0919
609.280660	609.280664	0.0060
962.430130	962.430230	0.1037

Observed Mass of Unknown: 595.16572

Empirical Formula Search Result:  $C_{27}H_{30}O_{15} [ +H ] +$

Mass: 595.16575

Mass Error: 0.04 ppm

Proposed Metabolite:  $C_{15}H_{10}O_6$  – Rhamnoglucoside  
 (present in flowers of grapefruit)

5

Comments: Figures 16 and 17 and Table IX show how our technology provides meaningful information that would otherwise not be obtained. In this example the researcher thought that he knew the primary color component in tobacco flowers (C<sub>15</sub>H<sub>10</sub>O<sub>6</sub>-Glucoside) but our analysis showed that the primary color component in tobacco flowers is actually the rhamnoglucoside. This illustrates the power of being able to identify unknown components after analysis. No other technology is currently available to provide this type of analysis.

10

Table X Illustration of the number of metabolites monitored in strawberry extracts.

Summary of Metabolites Observed from Different Extraction Methods and Ionization Conditions.

	Number of Unique Metabolites Observed			
	50/50	ACN	In Both	Total
ESI +	1143	1054	540	1657
ESI -	966	790	211	1545
APCI +	979	1431	615	1795
APCI -	898	1205	370	1733
<b>Total</b>	<b>3986</b>	<b>4480</b>	<b>1736</b>	<b>6730</b>

5

Table X and Figure 18 illustrate the comprehensive nature of our invention. Our technology allows for the comprehensive comparison of the metabolic profiles of organisms under varying environmental, genetic, and developmental conditions.

10

In this patent document, the word "comprising" is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. A reference to an element by the indefinite article "a" does not exclude the  
5 possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements.

It will be apparent to one skilled in the art that modifications may be made to the illustrated embodiment without departing from the spirit and scope of the invention as hereinafter  
10 defined in the Claims.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A Method for non-targeted complex sample analysis, comprising the steps of: providing  
5 a known molecule database (16) containing identifying data of known molecules; introducing a complex sample containing multiple unidentified molecules into a Fourier Transform Ion Cyclotron Mass Spectrometer (FTMS) (12) to obtain identifying and quantitation data regarding the molecules in the complex sample; comparing the collected data regarding the molecules in the complex sample with the identifying data of known  
10 molecules in order to arrive at the identification of the molecules in the sample; and the creation of a non-targeted metabolite database from all the identifying and quantitation data collected from the complex sample.
2. The method as defined in Claim 1, the complex sample being a biological sample.  
15
3. The method as defined in Claim 1, the complex sample being a combinatorial chemistry synthesis sample.
4. The method as defined in Claim 1, the identifying data being the experimentally  
20 determined empirical formula of the parent molecule and whose theoretical mass agrees to within 1.0 ppm relative error of the experimentally measured mass.
5. The method as defined in Claim 1, the identifying data being the accurate mass of the parent molecules experimentally determined with a relative error of determination less than  
25 1.0 ppm.
6. The method as defined in Claim 1, the identifying data being the accurate mass of the fragments of the parent molecules experimentally determined with a relative error of determination less than 5.0 ppm.  
30
7. The method as defined in Claim 1, the identifying data being the experimentally determined empirical formula of the fragment molecules of the parent molecules and

whose theoretical mass agrees to within 5.0 ppm relative error of the experimentally measured mass.

8. The method as defined in Claim 1, the quantitation data being the relative and/or  
5 absolute intensity of the parent molecule.

9. The method as defined in Claim 1, the quantitation data being the relative and/or absolute intensity of the fragment molecules.

10 10. The method as defined in Claim 1, the non-targeted metabolite database being organized to permit searching for known metabolites by accurate mass (defined as measured mass with less than 1.0 ppm relative error).

11. The method as defined in Claim 1, the non-targeted metabolite database being  
15 organized to permit searching for known metabolites by empirical formula.

12. The method as defined in Claim 1, the non-targeted metabolite database being organized to permit identification of metabolites by the accurate mass of the parent molecule.

20

13. The method as defined in Claim 1, the non-targeted metabolite database being organized to permit identification of metabolites by the empirical formula of the parent molecule.

25 14. The method as defined in Claim 1, the non-targeted metabolite database being organized to permit identification of metabolites by the empirical formulas of the fragments of the parent molecule.

15. The method as defined in Claim 1, the non-targeted metabolite database being  
30 organized to permit identification of metabolites by the accurate masses of the fragments of the parent molecule.

16. The method as defined in Claim 1, the non-targeted metabolite database being organized to permit the comparison of two samples to each other such that the relative intensity, presence, and/or absence of each metabolite is determined.
- 5 17. The method as defined in Claim 1, the non-targeted metabolite database being organized to permit the comparison of one or more “test” samples to a “control” sample such that the intensity, presence, and/or absence of the metabolites present in the “test” samples can be determined relative to the control sample and other test samples.
- 10 18. The method as defined in Claim 1, 16, or 17, the non-targeted metabolite database being organized to permit for the sorting, presenting and reporting of the data in ascending or descending order of the relative intensities determined.
- 15 19. The method as defined in Claim 1, 16, or 17, the non-targeted metabolite database being organized to permit for the sorting, presenting and reporting of the data according to the accurate mass of the fragments of the parent molecules.
- 20 20. The method as defined in Claim 1, 16, or 17, the non-targeted metabolite database being organized to permit for the sorting, presenting and reporting of the data according to the empirical formulas of the fragments of the parent molecules.
- 25 21. The method as defined in Claim 16, 17, 18, 19, 20, the correlation of the data contained within the non-target metabolite database from biological samples from a genetically modified “test” organism and its non genetically modified “control” organism with gene expression data from same said organisms for the purpose of determining the function of the genes affected by the genetic modification.
- 30 22. The method as defined in Claim 16, 17, 18, 19, 20, the correlation of the data contained within the non-target metabolite database from biological samples from an organism exposed to a “test” environment and a “control” environment with gene expression data from same said organism under same said conditions for the purpose of determining the function of the genes affected by the test environment.



23. The method as defined in Claim 22, the test environment is deemed to be any internal or external force imparted on the organism that may have an impact on its function. Examples include but are not limited to: exposure to or withdrawal from drug, pesticide, nutrient, or other chemical entity, weather conditions such as drought, frost, heat, psychological conditions such as stress.

24. The method as defined in Claim 16, 17, 18, 19, 20, the correlation of the data contained within the non-target metabolite database from biological samples from an organism at different stages of its development with gene expression data from same said organism at same said stages of its development for the purpose of determining the function of the genes affected by the changes in development of the organism.

25. The method as defined in Claim 16, 17, 18, 19, 20, the correlation of the data contained within the non-target metabolite database from biological samples from a healthy organism and diseased organism with gene expression data from same said organisms for the purpose of determining the function of the genes affected by the disease state of the organism.

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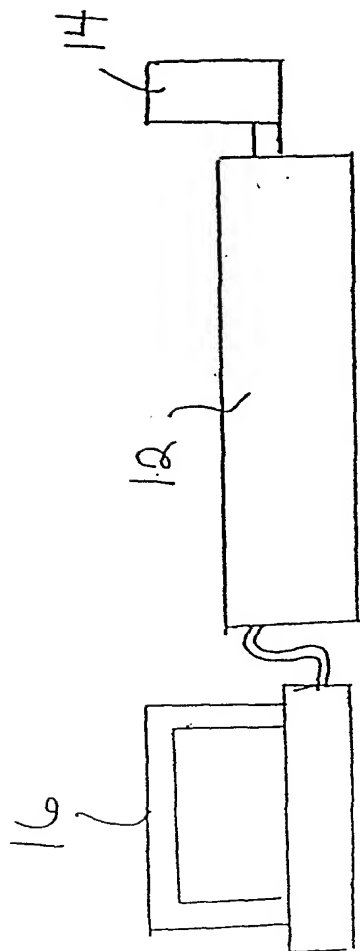


FIGURE 1

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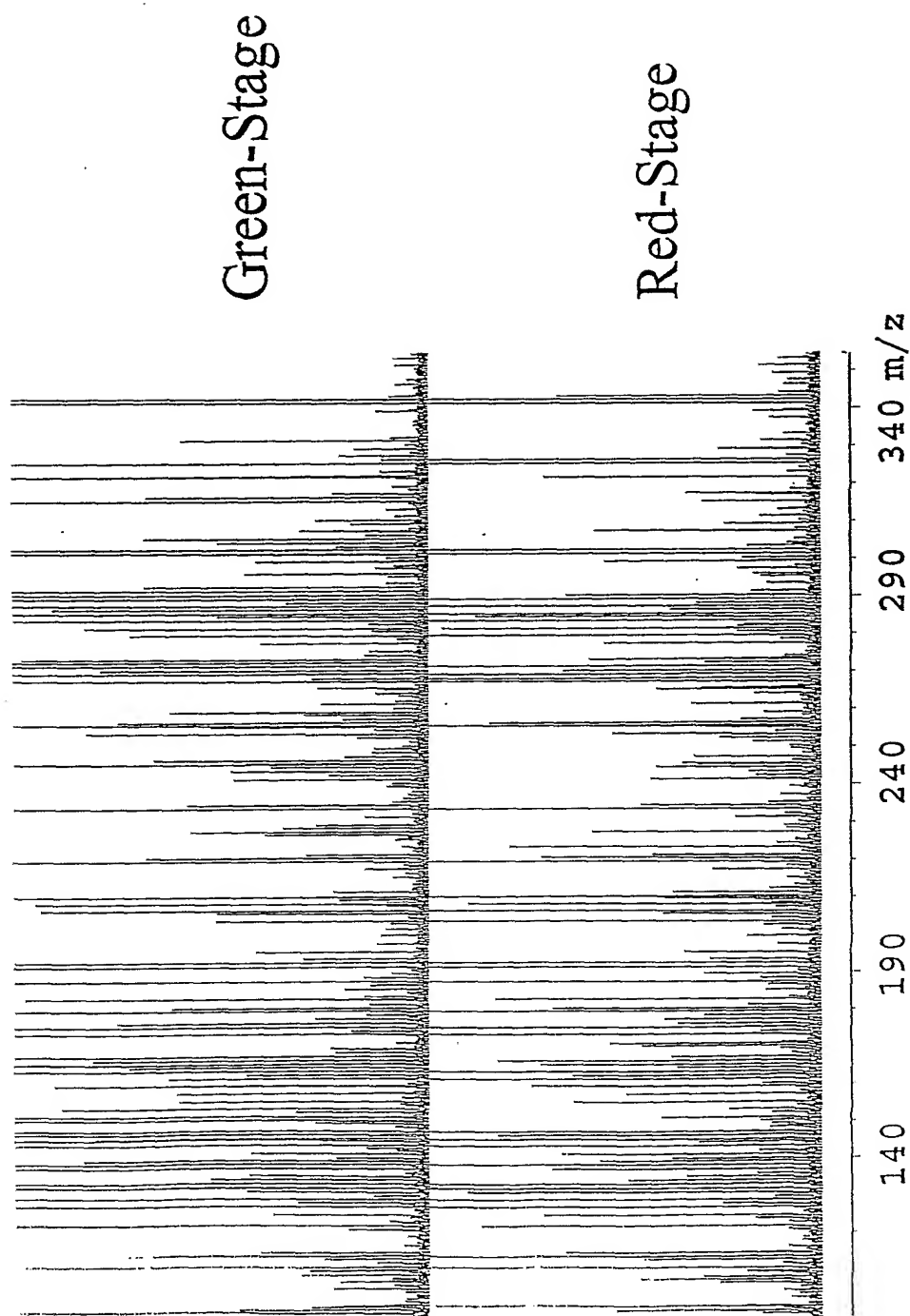


FIGURE 2

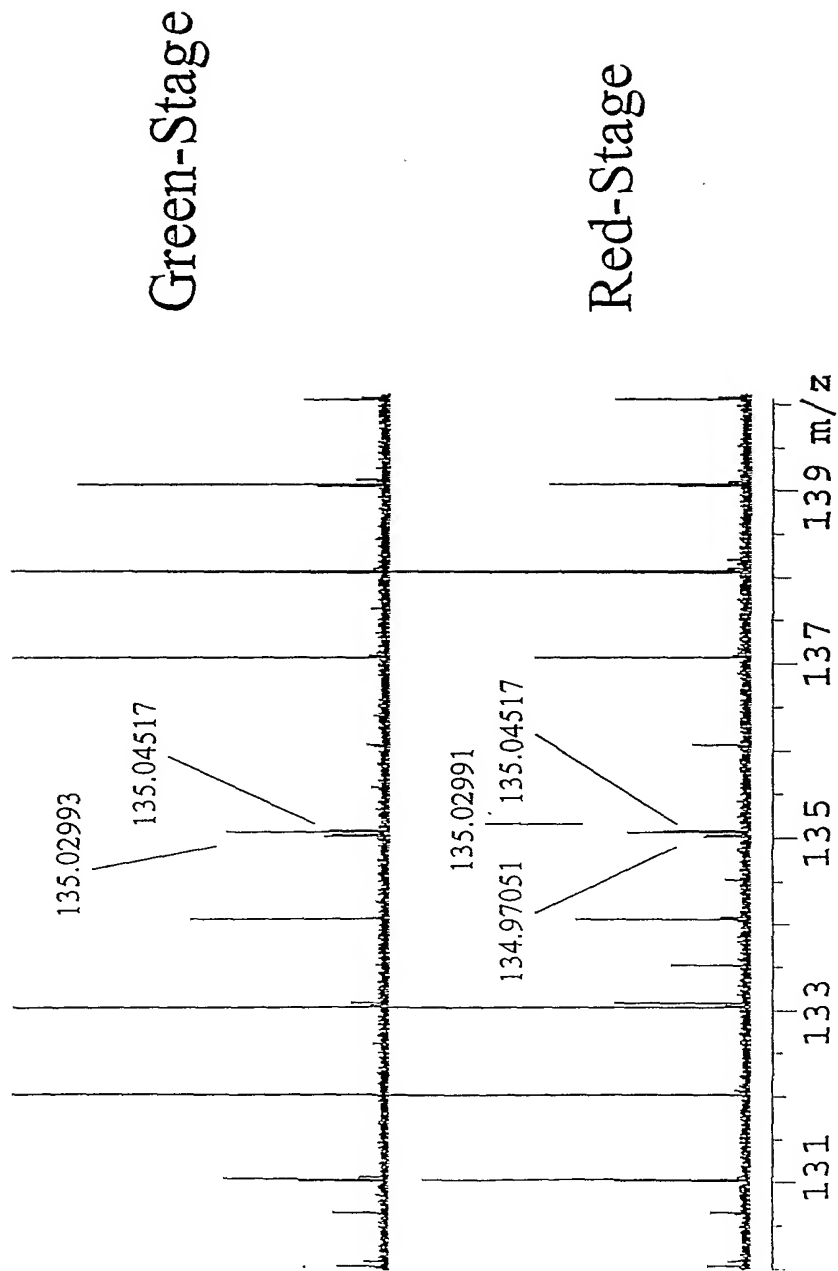


FIGURE 3

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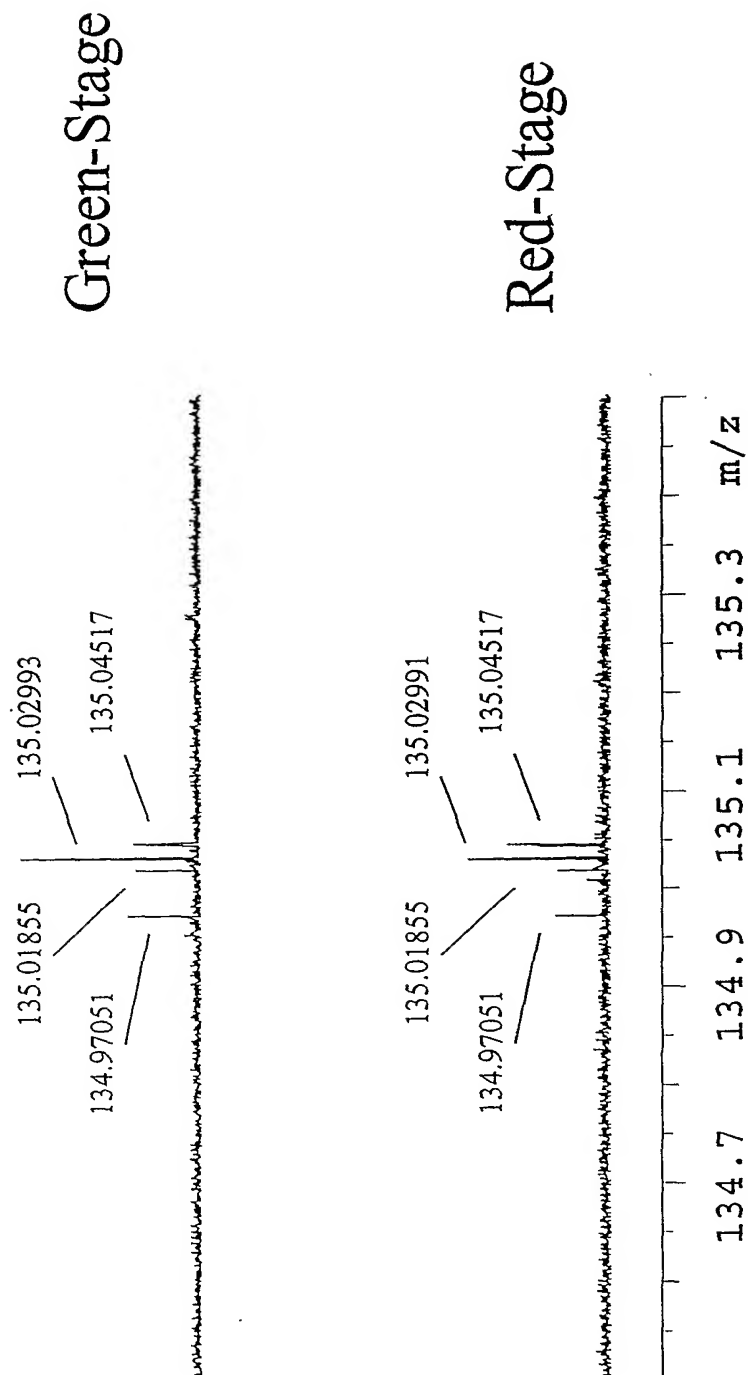


FIGURE 4

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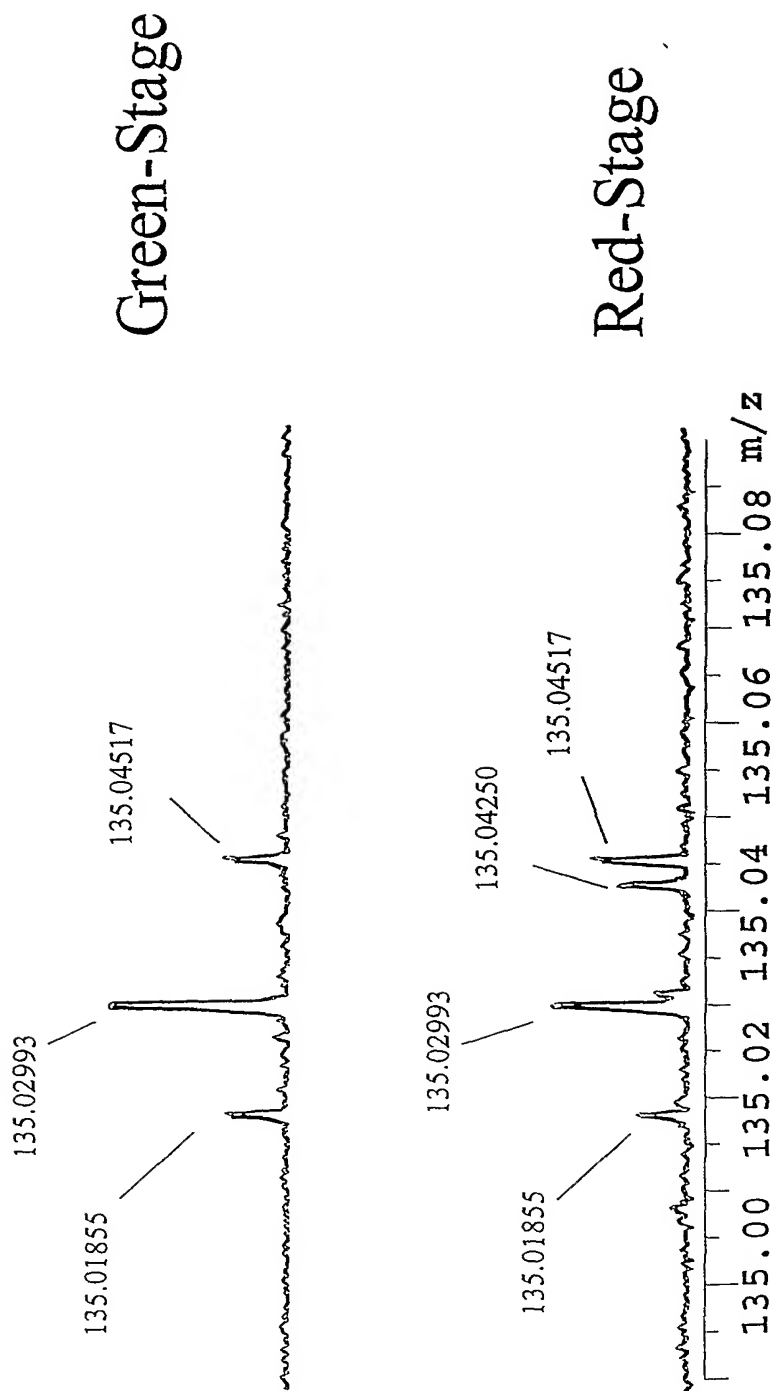


FIGURE 5

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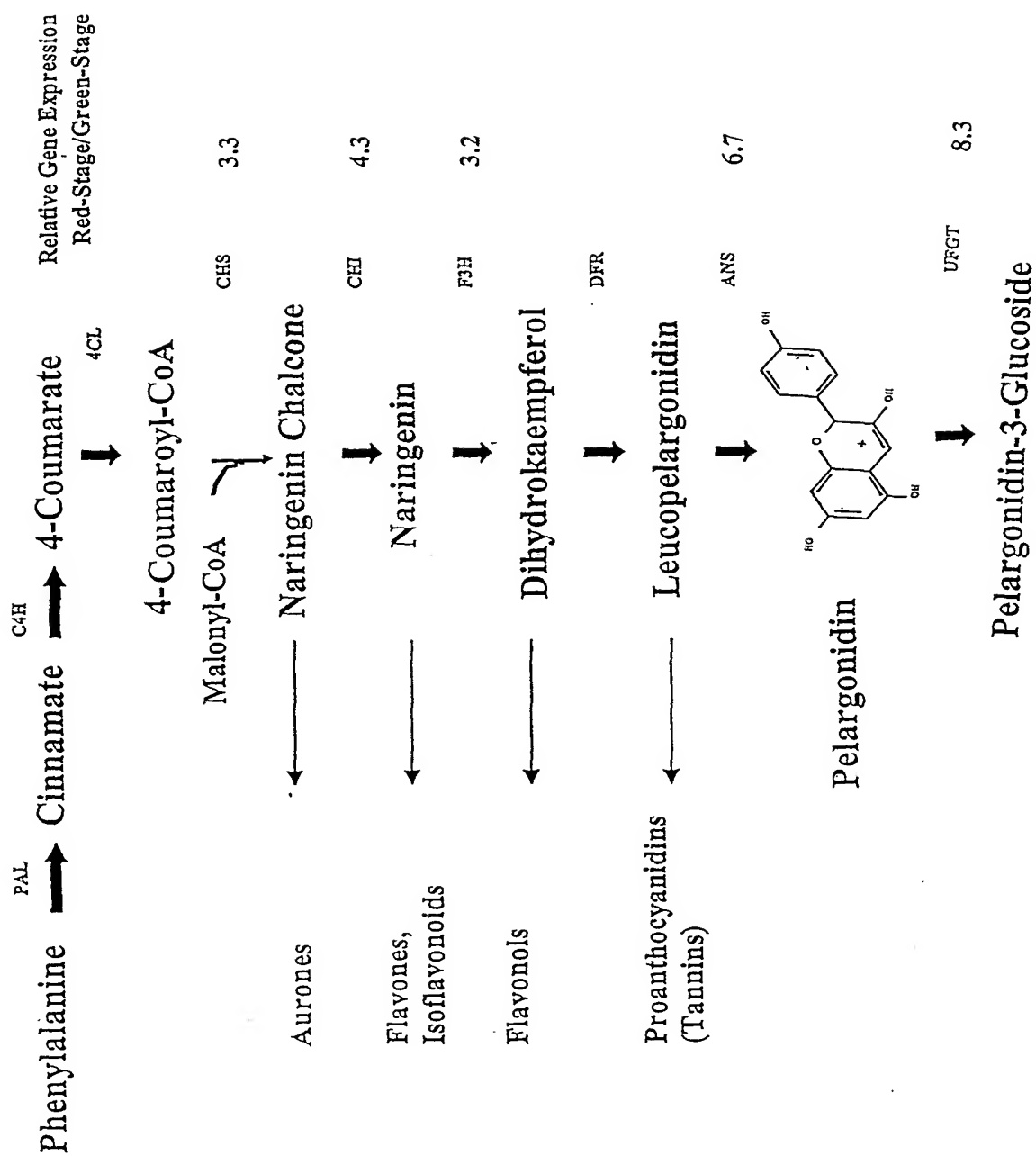


FIGURE 6

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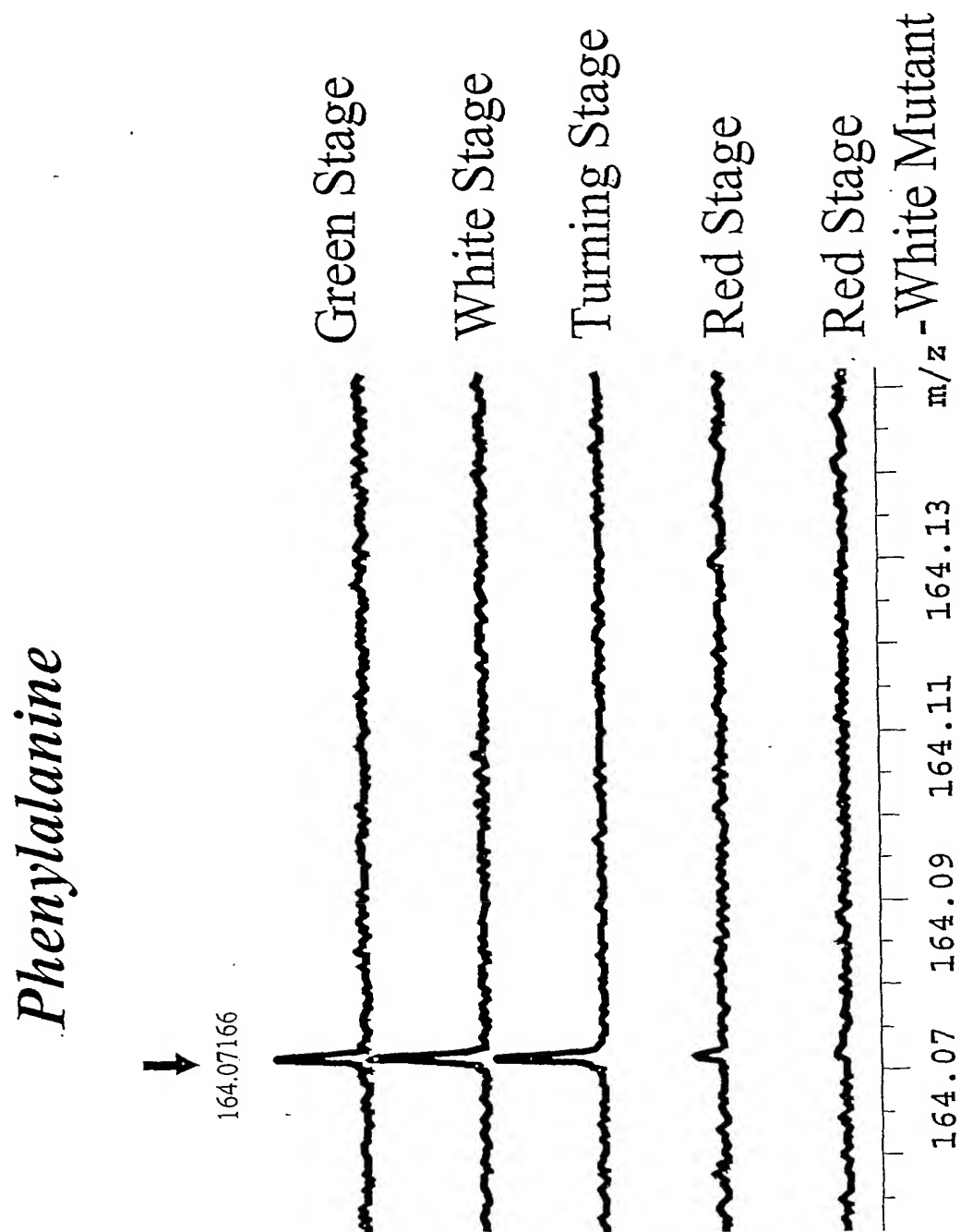


FIGURE 7



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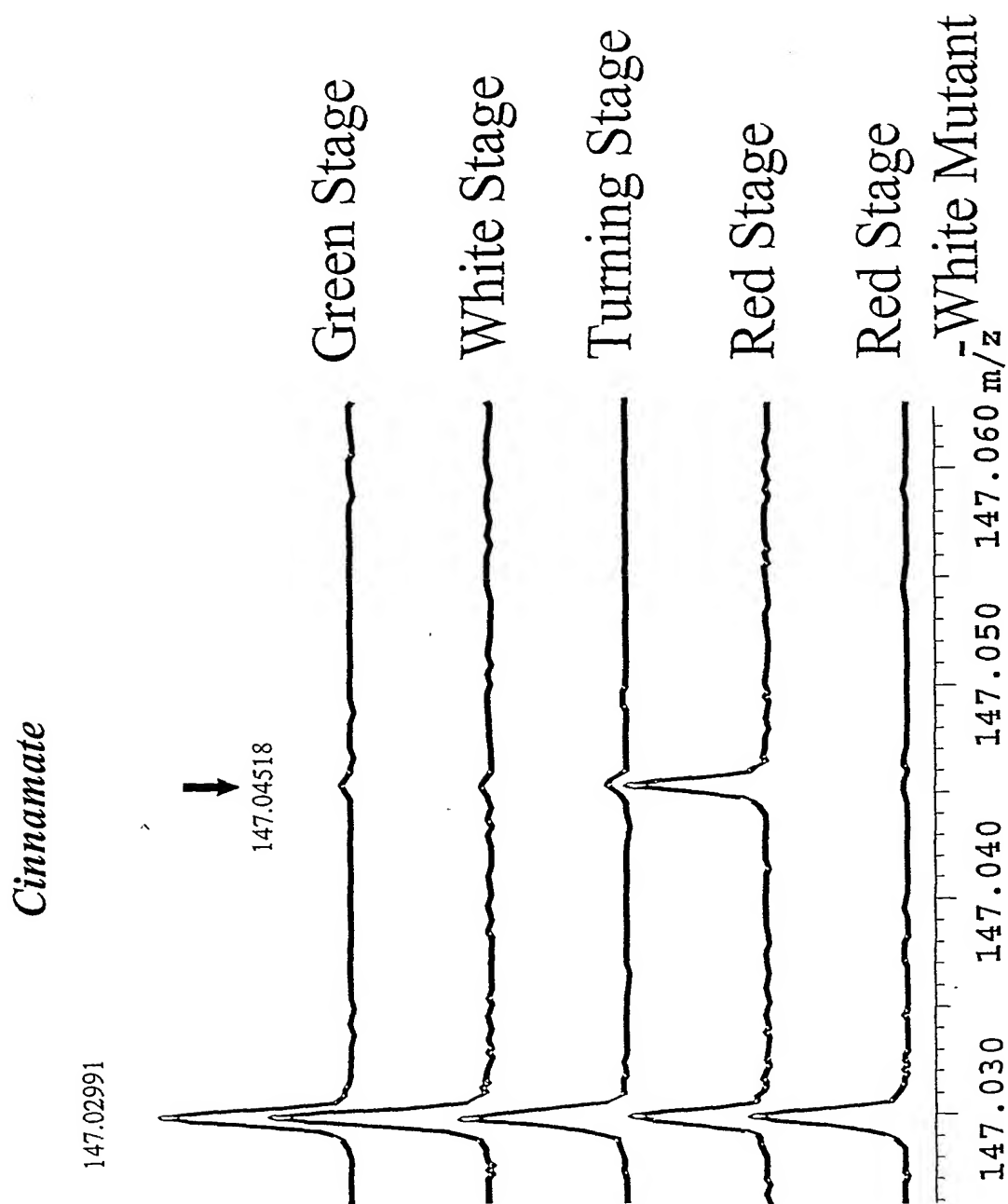


FIGURE 8

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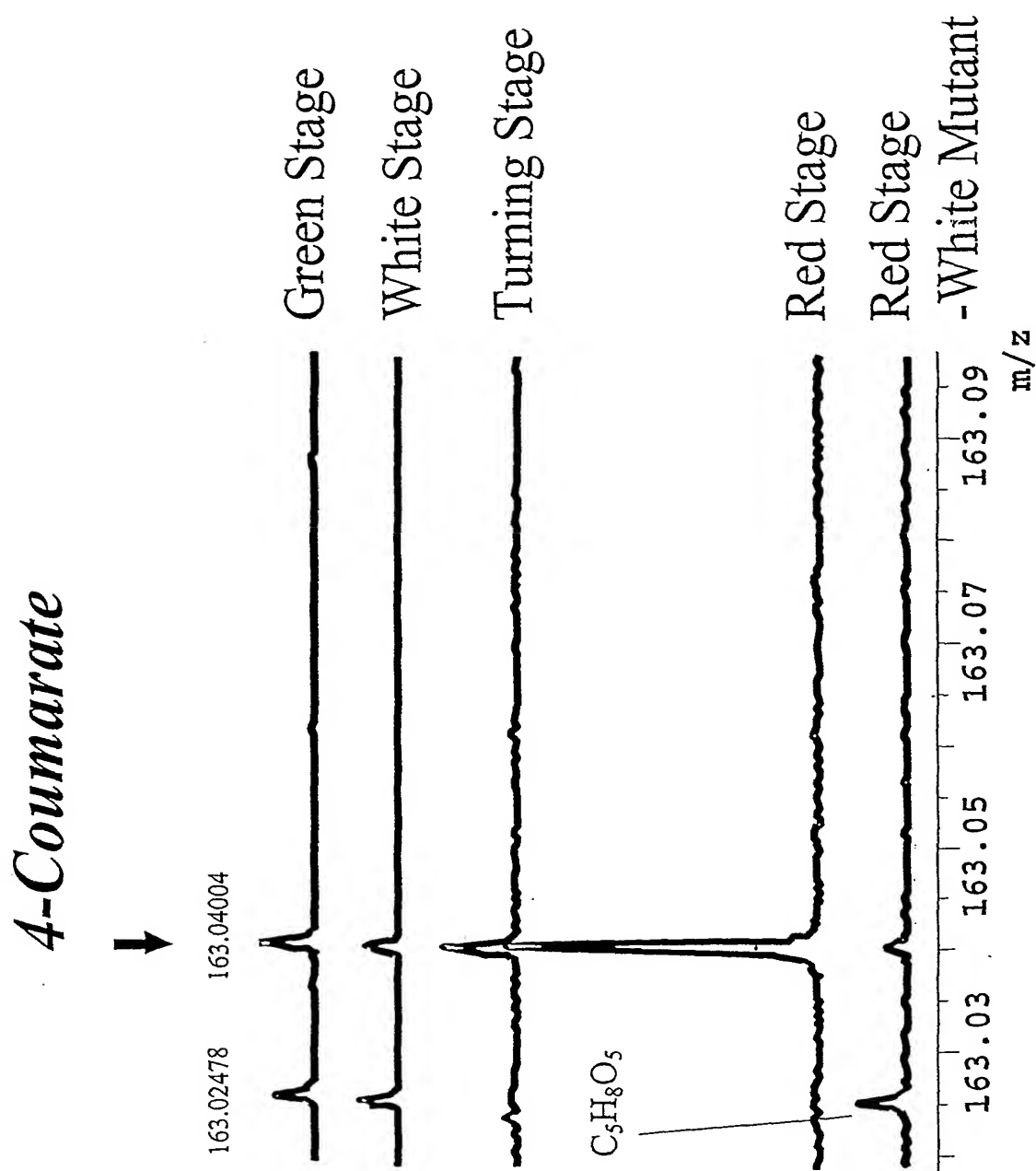


FIGURE 9

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# Naringenin

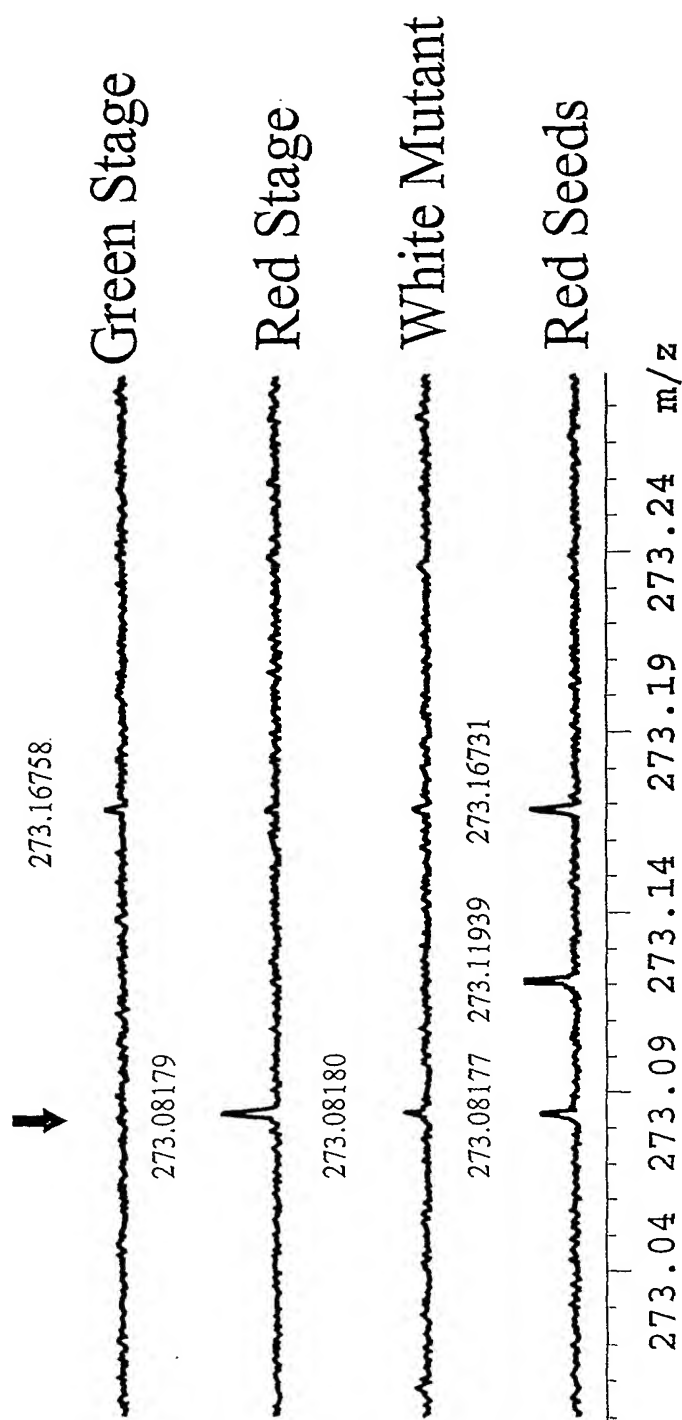


FIGURE 10

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# Pelargonidin

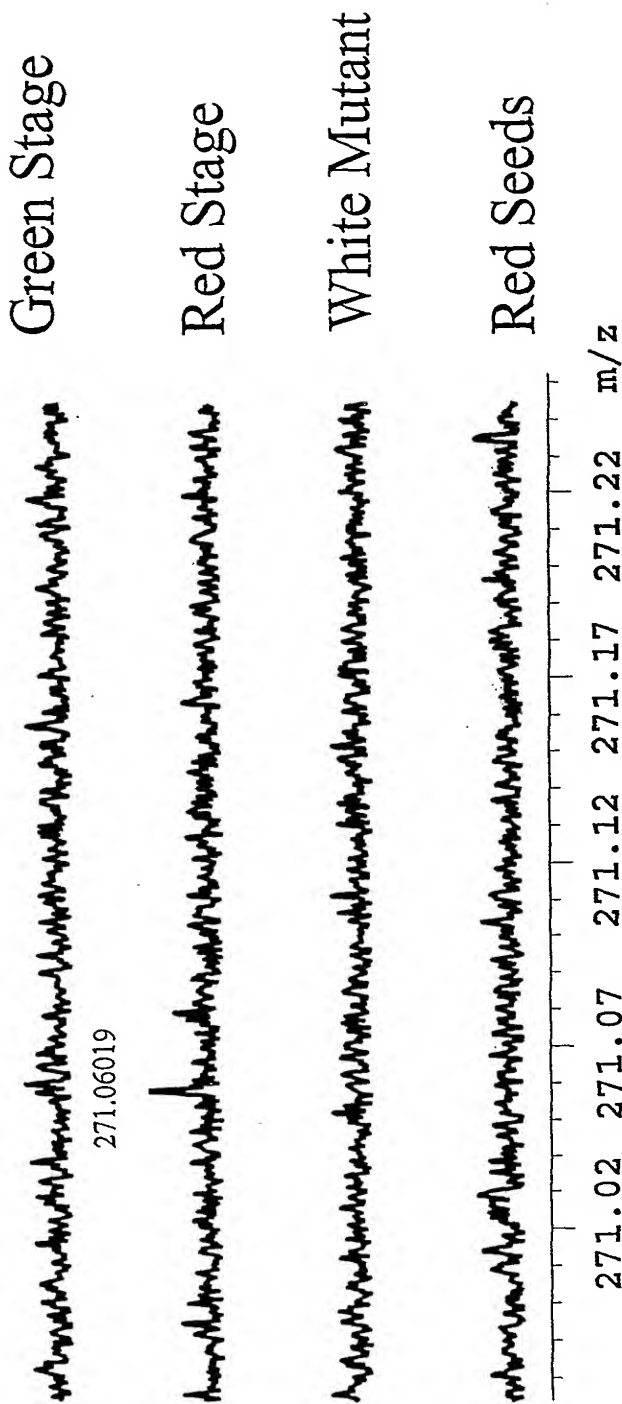


FIGURE 11

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# Pelargonidin-3-Glucoside

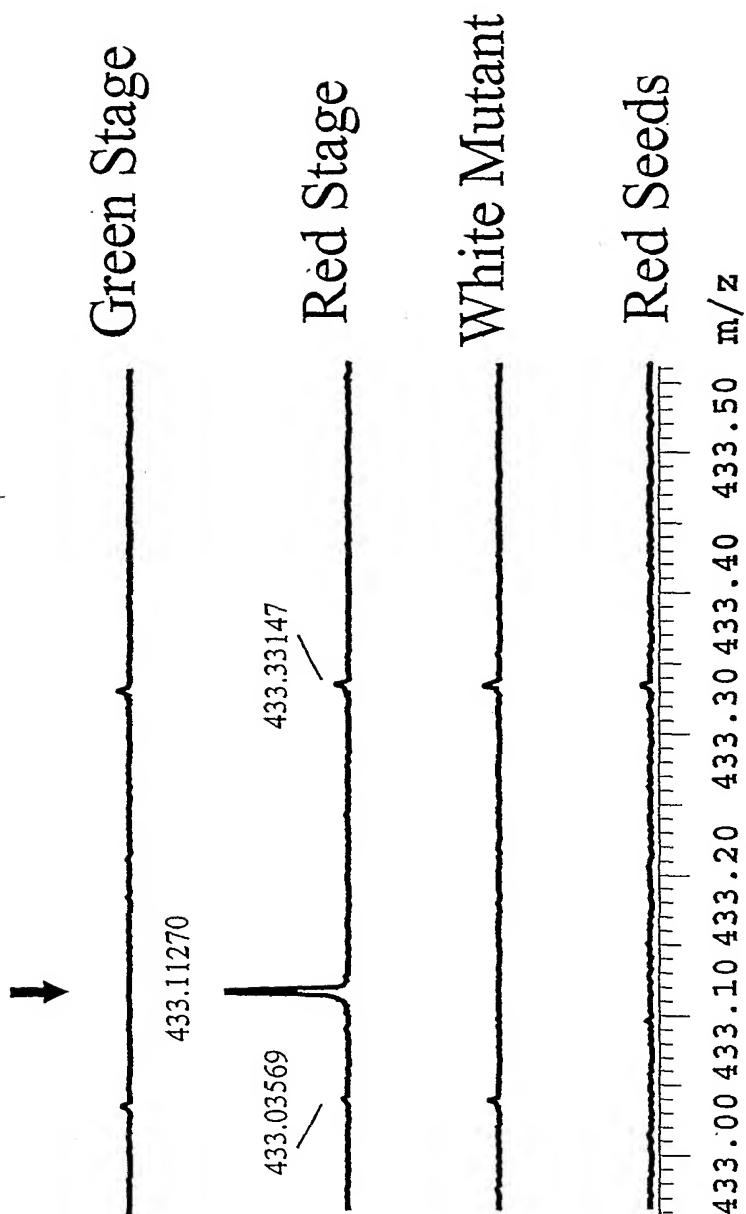


FIGURE 12

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# 3-Methylthiobutyl Glucosinolate

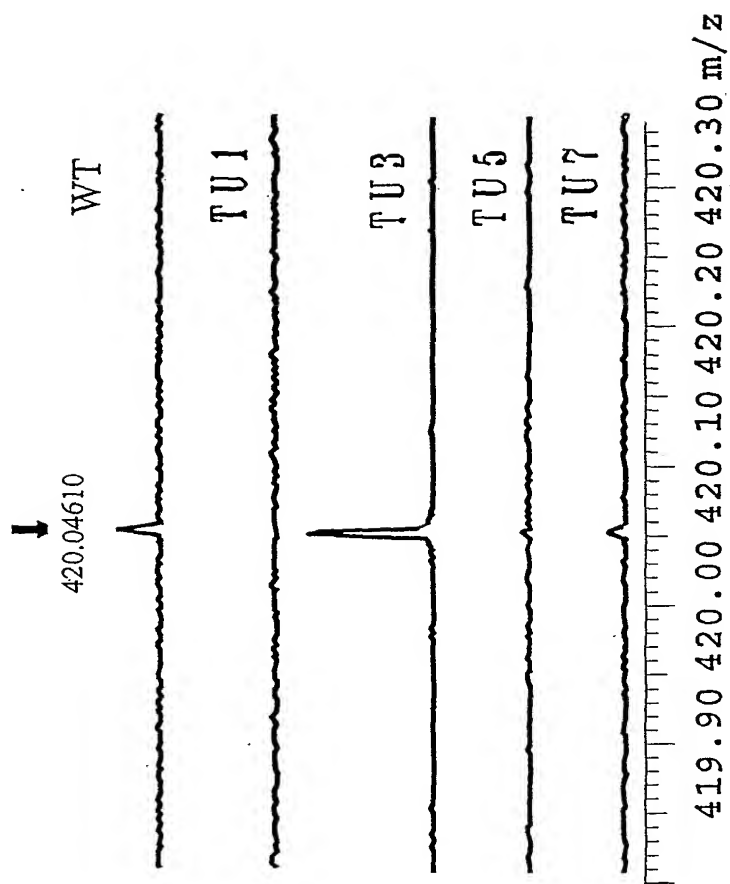


FIGURE 13

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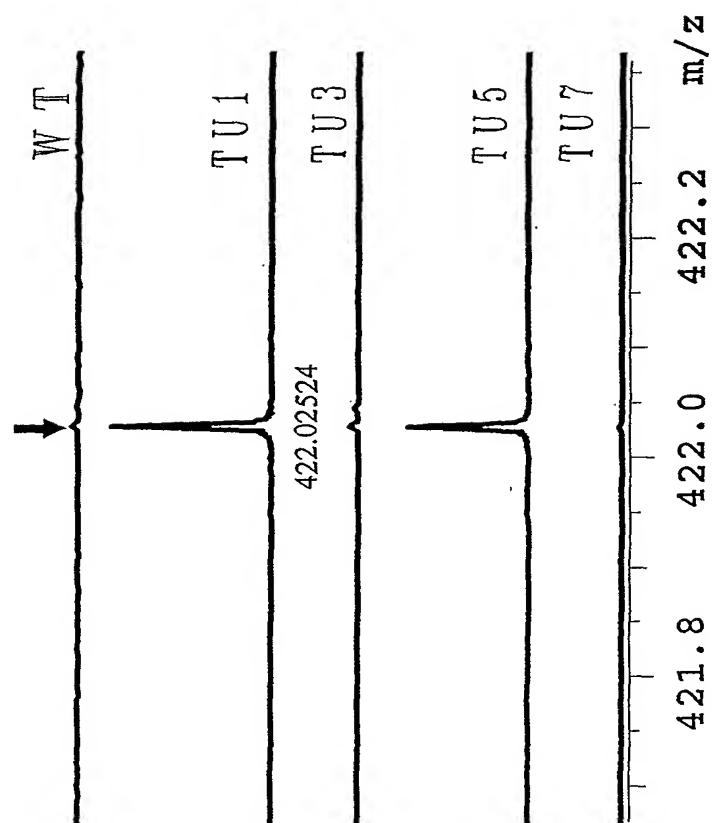
*3-Methylsulphinypropyl Glucosinolate*

FIGURE 14

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### 3-Methylsulphinylnheptyl Glucosinolate

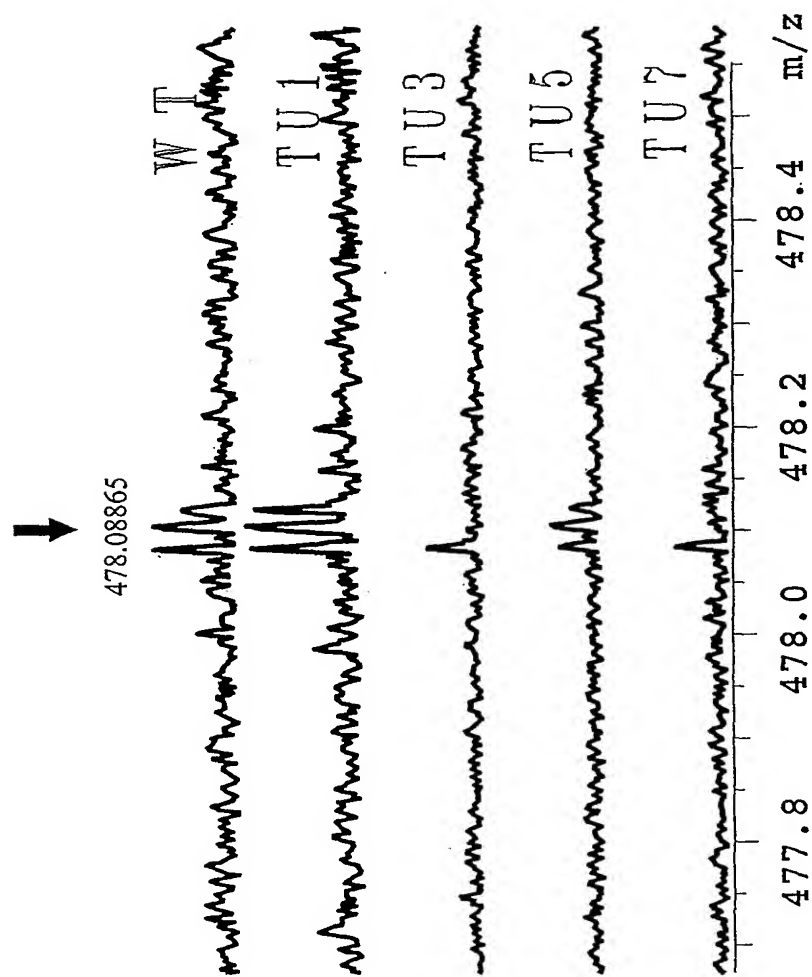
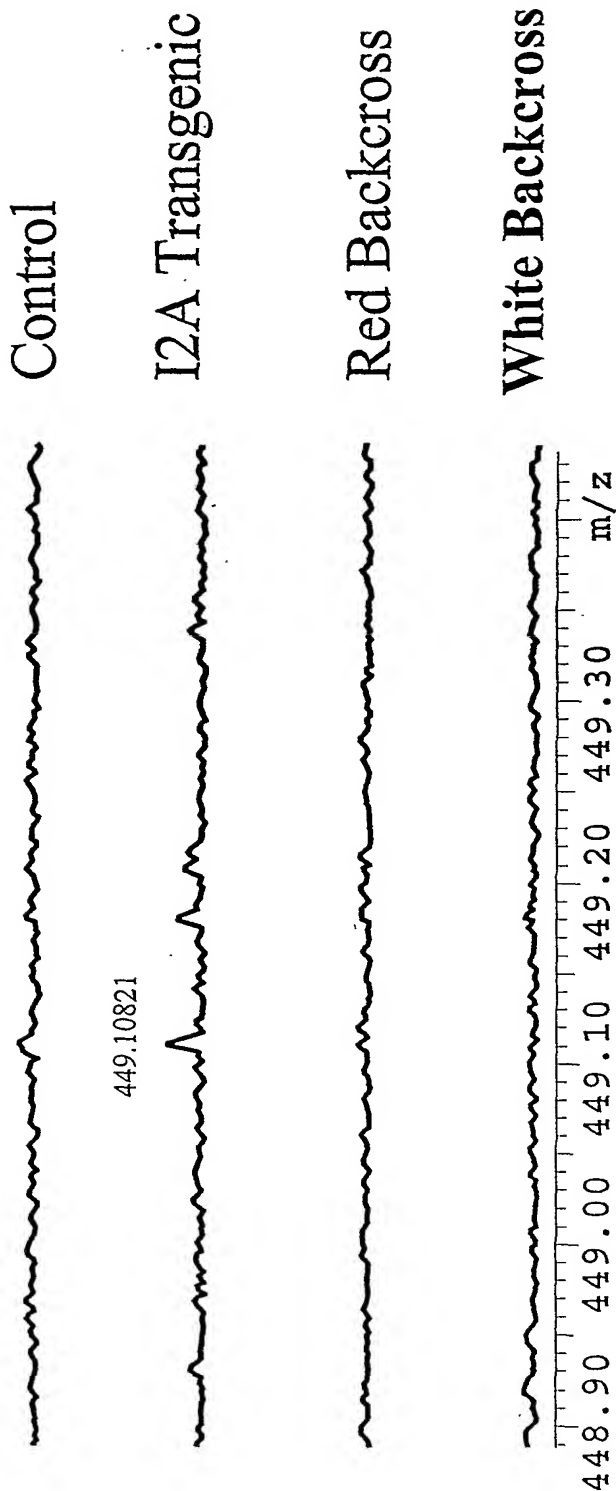


FIGURE 15



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# *Red vs. White Tobacco*



$C_{15}H_{10}O_6$  - Glucoside (Theoretical Mass = 449.1078)  
Expected - but not Observed

FIGURE 16

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# *Red vs. White Tobacco*

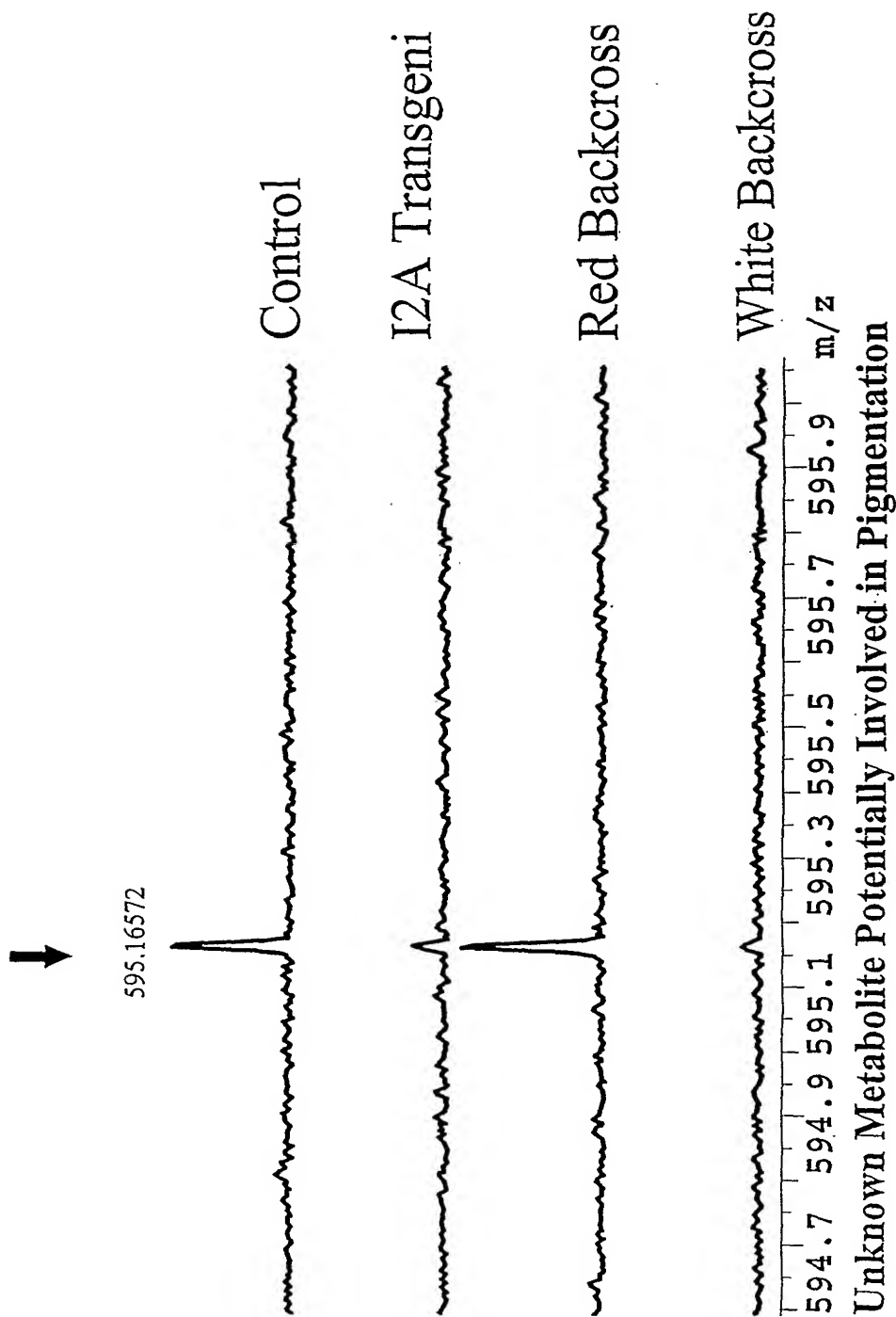
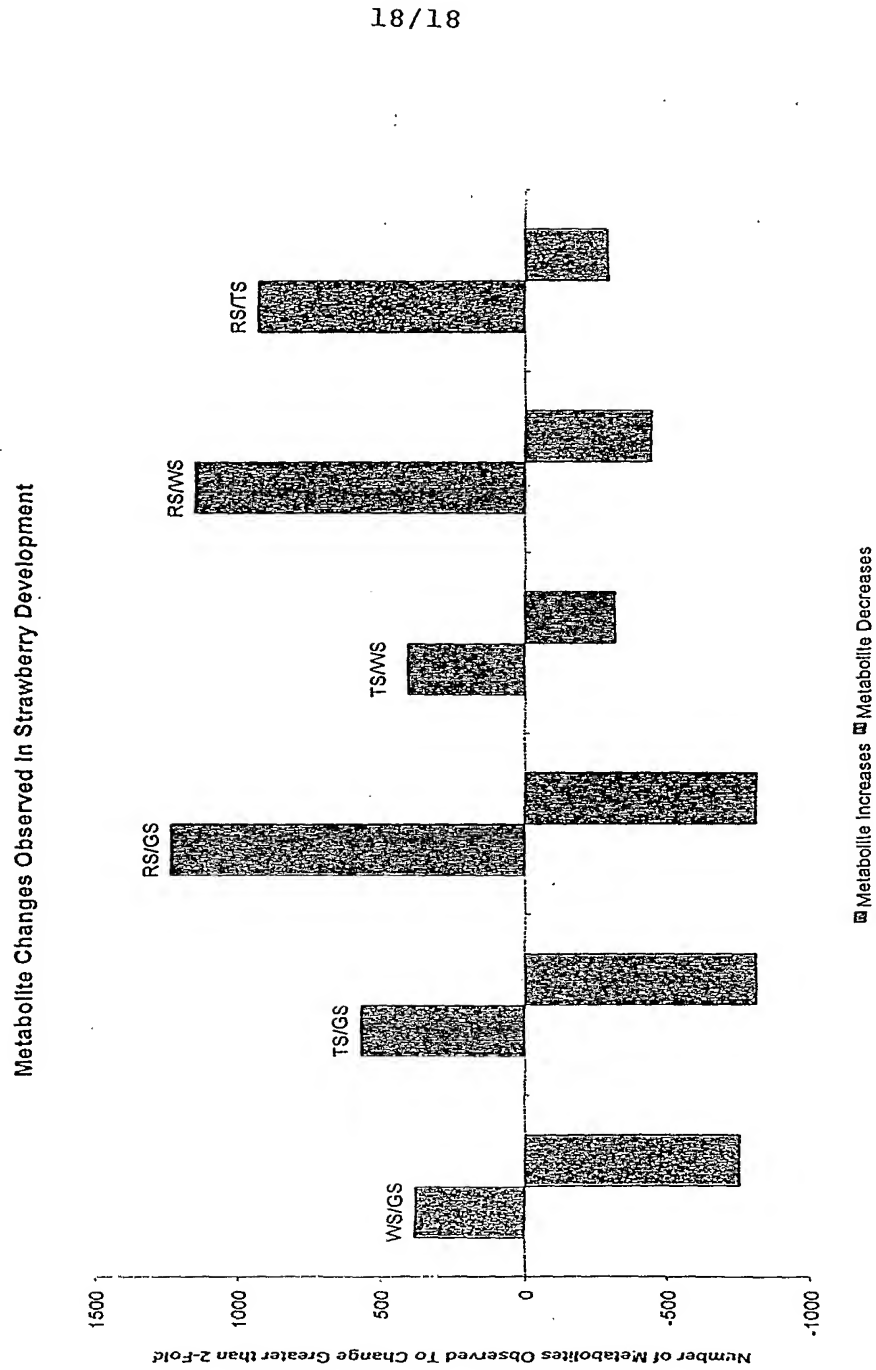


FIGURE 17



GS-Green Stage; WS-White Stage; TS-Turning Stage; RS-Red Stage

**FIGURE 18**